The effect of antirheumatic drugs on prostaglandin synthetase from rheumatoid tissue.

Crook, D.

Award date:
1977

Awarding institution:
University of Bath

THE EFFECT OF ANTIRHEUMATIC DRUGS ON PROSTAGLANDIN SYNTHETASE FROM RHEUMATOID TISSUE

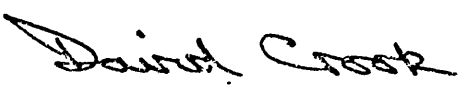
Submitted by

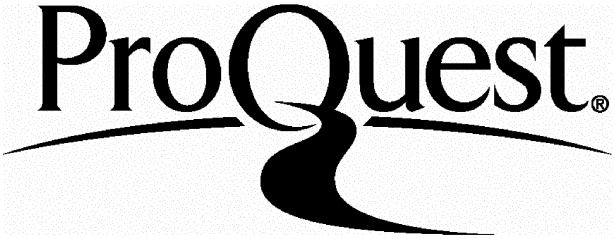
D. Crook, B.Sc. (Hons.)

for the Degree of Doctor of Philosophy

of the University of Bath

1977





SUMMARY

The proposal of Vane (1971) that aspirin-like drugs exert their pharmacological actions via inhibition of prostaglandin (PG) biosynthesis is now widely accepted. It was the object of the present work to prepare PG synthetase from human rheumatoid synovial tissue in order to study its basic biochemical properties, and to study the interaction(s) between the enzyme and some of the aspirin-like drugs commonly used in the therapy of the rheumatic diseases.

Using a radiometric technique PG synthetase activity was measured *in vitro* in the microsomal fraction of 31 synovial tissues taken from 27 patients with rheumatoid arthritis. Its biochemical properties and inhibition *in vitro* by low concentrations of aspirin-like drugs suggested that it did not differ radically from the well-studied enzymes prepared from animal tissues.

While the enzyme preparation from patients receiving indomethacin, naproxen or ibuprofen therapy possessed considerable activity *in vitro*, preparations from patients receiving aspirin, even in low doses, were incapable of PG synthesis. Aspirin may therefore be unique in being an irreversible inhibitor of the enzyme.

A correlation was found to exist between the potency of the aspirin-like drugs as inhibitors of synovial PG synthetase *in vitro* and their known therapeutic potencies. The significance of interesting anomalies such as salicylic acid is discussed.

Studies of the effects of copper and copper aspirin showed interesting quantitative and qualitative effects *in vitro* which may have therapeutic potential.

Studies with human peripheral leucocytes showed that platelets contain a high level of synthetase activity and a comparison of the effects of aspirin and indomethacin, both *in vitro* and *in vivo*, confirmed the results obtained with synovial preparations.

The use of /¯acetyl-^{3}H¯/ aspirin of high specific activity showed the drug to be capable of acetylating a particulate fraction protein from both platelet and synovial preparations. The possibility that this protein is the cyclo-oxygenase component of the enzyme complex is discussed.

C O N T E N T S

ACKNOWLEDGEMENTS

I would like to thank Professor R.J. Ancill for providing the facilities to carry out this study and giving me the opportunity to work in the School of Pharmacy and Pharmacology.

I would also like to thank my supervisor, Dr. A.J. Collins for his constant enthusiasm and encouragement throughout three years of work. Thanks are also due to Dr. K.A. Brown for his active participation in some of the experiments involving peripheral leucocytes, and to Dr. A.J. Rose for helpful advice in the early stages of this project.

Finally my sincere thanks are extended to the hospitals and staff of the Bath Health District for their co-operation and assistance in providing human tissues used in this work.

PUBLICATIONS

A substantial part of the work presented in this thesis has been published in the following papers :-

Crook, D. and Collins, A.J. (1975) Prostaglandins, 9, 857-865. (Prostaglandin synthetase activity from human rheumatoid synovial tissue and its inhibition by non-steroidal anti-inflammatory drugs).

Crook, D., Collins, A.J. and Rose, A.J. (1976). J.Pharm.Pharmac. 28, 535. (A comparison of the effect of flurbiprofen on prostaglandin synthetase from human rheumatoid synovium and enzymatically active animal tissues).

Crook, D., Collins, A.J., Bacon, P.A. and Chan, R. (1976). Ann.rheum.Dis. 35, 327-332. (Prostaglandin synthetase activity from human rheumatoid synovial microsomes. Effect of 'aspirin-like' drug therapy).

Collins, A.J., Crook, D., Chan, R. and Bacon, P.A. (1976). In: 'The Role of Prostaglandins in Inflammation'. Ed. Lewis, G.P. p. 138-147. (Prostaglandin synthetase activity in synovial tissue from patients with rheumatoid arthritis, after therapy with aspirin-like drugs).

Crook, D. and Collins, A.J. (1977). Ann.rheum.Dis. In press. (A comparison of the effects of aspirin and indomethacin on human platelet prostaglandin synthetase).

"It is now apparent that prostaglandins are implicated in many biological mechanisms, both physiological and pathological."

Horton, 1976.

"Perhaps prostaglandins are just a way of removing lipids capable of forming damaging peroxides : they would then be seen as metabolic waste, of only slightly more value to the animal than is morphine to the poppy or digitalis to the foxglove."

Brocklehurst & Dawson, 1974.

"The prostaglandins have the ability to stimulate as well as to sow confusion; privately, we are convinced that the role of prostaglandins is to make people go back to school and learn their subjects a little better."

Ramwell & Pharriss, 1972.

CHAPTER ONE

GENERAL INTRODUCTION

A. PROSTAGLANDINS

1.1 Historical

Studies by Goldblatt (1933) and von Euler (1935) showed that human seminal plasma and extracts of the vesicular gland of sheep contained a substance possessing vasodepressor and smooth muscle stimulating activity which, owing to its occurence in extracts of the prostate and vesicular gland was called prostaglandin. Preliminary characterisation showed that prostaglandin had the properties of a fatty acid, being readily extracted into lipid solvents from an acidified aqueous solution, and freely soluble in aqueous alkali. It was further established that prostaglandin was a nitrogen-free compound, and that it probably possessed hydrophilic groups and some degree of unsaturation. The formation of a stable, water-soluble barium salt permitted some purification of the crude extract, but analytical techniques available at the time were inadequate for further elucidation of the structure of prostaglandin.

The development of chromatographic techniques during the 1950's provided a powerful new tool for the purification and separation of crude biological extracts, and in 1960 Bergström and Sjövall succeeded in isolating an active compound from sheep glands in crystalline form. Furthermore, it soon became apparent that 'prostaglandin' was not a single substance, but consisted of a family of closely related chemical structures. In the space of the next four years Bergström's group were able to separate 13 distinct compounds, all derivatives of a parent substance given the trivial name prostanoic acid.

Elegant work involving micro-analytical degradations and transformations elucidated the various structures of the prostaglandins,

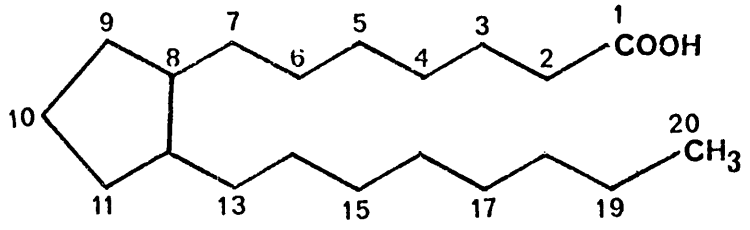
final confirmation being achieved by X-ray crystallography (Abrahamsson, Bergström and Samuelsson, 1962) and mass-spectrometry (Bergström and others, 1962). Although the early work on prostaglandins established that high concentrations existed in seminal vesicular glands and in the seminal fluid of man and some animals, later work showed virtually all tissues and cell types studied to be capable of prostaglandin biosynthesis, thus raising the possibility that prostaglandins might have a more general significance than at first anticipated.

1.2 Chemistry

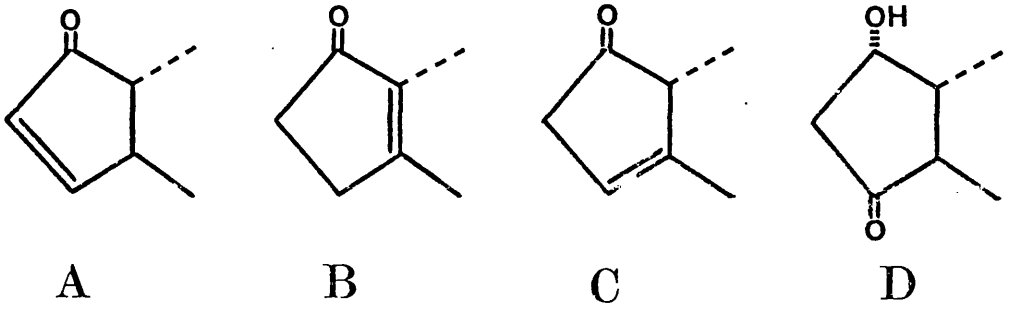
All prostaglandins (PG's) are derivatives of a C_{20} mono-carboxylic acid, which has been given the trivial name prostanoic acid (Fig 1.1). It is not a naturally occuring compound. The main families or series of prostaglandins are denoted by a letter e.g. prostaglandin F (universally abbreviated to PGF). Eight series have been recognised to date, extending alphabetically from PGA to PGH inclusive. Each series is distinguished by the type and position of various functional groups in the molecule, usually within the cyclopentane ring, i.e. on carbon atoms 8-12. PGG is unique in possessing a hydroperoxy group at C-15, all others having a hydroxyl group in this position.

Chemical conversion between some of the different series is readily achieved. Thus, treatment of F-type prostaglandins with acid or base yields A or B-type prostaglandins respectively and this can be used to assay PGE, as both PGA and PGB are chromophores exhibiting characteristic UV absorption maxima at 217nm and 278nm respectively. Chemically, the A-type prostaglandins are dehydration products of the E series, in which the 11-hydroxyl group has been lost and a double bond formed between C-10 and C-11. The B-type

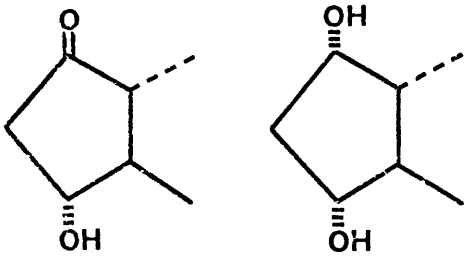
Fig. 1.1. Numbering scheme for prostanoic acid and chemical structures of the prostaglandins



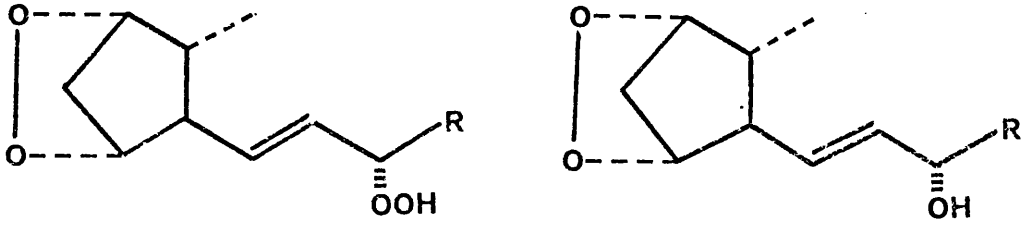
PROSTANOIC ACID



A B C D



E F



G H

prostaglandins and isomers of PGA in which the double bond is shifted from Δ^{10-11} to Δ^{8-12}, via the relatively unstable Δ^{11-12} compound PGC. Clearly, extremes of pH must be avoided during the extraction and separation of prostaglandins in order to prevent the formation of PGA or PGB artefacts from PGE compounds.

Treatment of PGE (or its isomer PGD in which the 9-oxo and 11-OH groups are reversed) with a reducing agent such as sodium borohydride yields a mixture of PGF_{α} and PGF_{β}, the α and β referring to whether the hydroxyl substituent at C-9 is below (α) or above (β) the plane of the cyclopentane ring. Conversion of the relatively unstable PGG and PGH (the so-called "cyclic endoperoxides") to PGF is readily achieved with a reducing agent such as stannous chloride.

Within each prostaglandin family or series several members are possible, differing only in the number of double bonds present in their side chains, and these are designated by subscript numbers e.g. PGE_1, PGE_2 etc. Members of the "1" series have a single Δ^{13-14} trans double bond. Members of the "2" series have an additional Δ^{5-6} cis double bond, while members of the "3" series have a further Δ^{17-18} cis double bond. The degree of unsaturation of a PG is determined by the number of double bonds present in the fatty acid precursor of each series; thus the fatty acid precursors of the 1, 2 and 3 series are all-cis-8, 11, 14-eicosatrienoic acid (dihomo-γ-linolenic acid), all-cis-5, 8, 11, 14-eicosatetraenoic acid (arachidonic acid) and all-cis-5, 8, 11, 14, 17-eicosapentaenoic acid respectively.* There is no evidence that interconversion between prostaglandins with differing degrees of unsaturation occurs either in vivo or in vitro,

---

*Pace-Asciak (1976) has recently described the formation of 6-keto-$PGF_{1\alpha}$ from arachidonic acid by homogenates of rat stomach.

e.g. PGE_1 to PGE_2 or vice versa.

In most tissues prostaglandins of the 2 series predominate due to the relative abundance of arachidonic acid compared with dihomo-γ-linolenic acid. Prostaglandins of the 3 series, though found in human and sheep semen and sheep vesicular gland, (PGE_3 and $PGF_{3\alpha}$) are difficult to isolate and are susceptible to auto-oxidation. Their biological properties have not been extensively investigated, though reports so far indicate that their properties are similar to the corresponding 2 series compounds.

1.3 Biosynthesis

The biosynthesis of prostaglandins from a C_{20} polyunsaturated ("essential") fatty acid was demonstrated simultaneously by two groups of workers (van Dorp and others, 1964; Bergström, Danielsson and Samuelsson, 1964). Aerobic incubation of homogenised sheep vesicular glands with tritium labelled 5, 8, 11, 14-eicosatetraenoic acid (arachidonic acid) gave a 20% yield of a compound having identical chemical, spectral and chromatographic properties to those of an authentic sample of PGE_2. The enzymatic conversion of 8, 11, 14-eicosatrienoic acid (dihomo-γ-linolenic acid) and 5, 8, 11, 14, 17-eicosapentaenoic acid to PGE_1 and PGE_3 respectively was subsequently achieved (Bergström and others, 1964; van Dorp and others, 1964b) using the same enzyme source.

The multi-enzyme complex, prostaglandin synthetase (E.C. 1.14.99.1) is found in the high-speed particulate ("microsomal") fraction of cells but may be solubilised with the use of suitable non-ionic detergents (Sih and Takeguchi, 1973). A preliminary resolution of the enzyme complex into its separate oxygenase and isomerase components has been achieved using DEAE-cellulose column chromatography of solubilised

bovine vesicular gland microsomes (Miamoto, Yamamoto and Hayaishi, 1974). The oxygenase component (Fraction I) itself exhibited two distinct enzyme activities; firstly, in the presence of appropriate co-factors such as haemoglobin and tryptophan, it was able to catalyse the formation of PGG_1 from dihomo-γ-linolenic acid and secondly to transform PGG_1 into PGH_1 (i.e. 15-hydroperoxy → 15-hydroxy). The isomerase component (Fraction II), in the presence of reduced glutathione was able to transform the cyclic endoperoxide PGH_1 into PGE_1. Recently Fraction I has been further purified by isoelectric focussing (Miyamoto and others, 1976) and provisionally named prostaglandin endoperoxide synthetase. A preliminary estimation of the molecular weight of the purified enzyme, by gel filtration on Sephadex G−200 in the presence of 0.2% Tween 20, gave a value of 300,000-350,000.

Many workers have used the microsomal fraction from tissues as an enzyme source for incubation studies as this gives a preparation of high specific activity and also effectively removes the prostaglandin metabolising enzymes present in the cytoplasmic fraction of cells.

Mechanism of PG biosynthesis

The separate steps involved in the biosynthesis of prostaglandins from polyunsaturated fatty acids have been reasonably well elucidated (Nugteren, Beerthuis and van Dorp, 1966; Hamberg and Samuelsson, 1967), though doubt still exists as to whether some of the transformations are achieved by enzymatic or non-enzymatic means. Although much of this early work was done with the PG synthetase system derived from sheep seminal vesicular glands, there is no reason to believe that biosynthesis takes place in other tissues by radically different pathways.

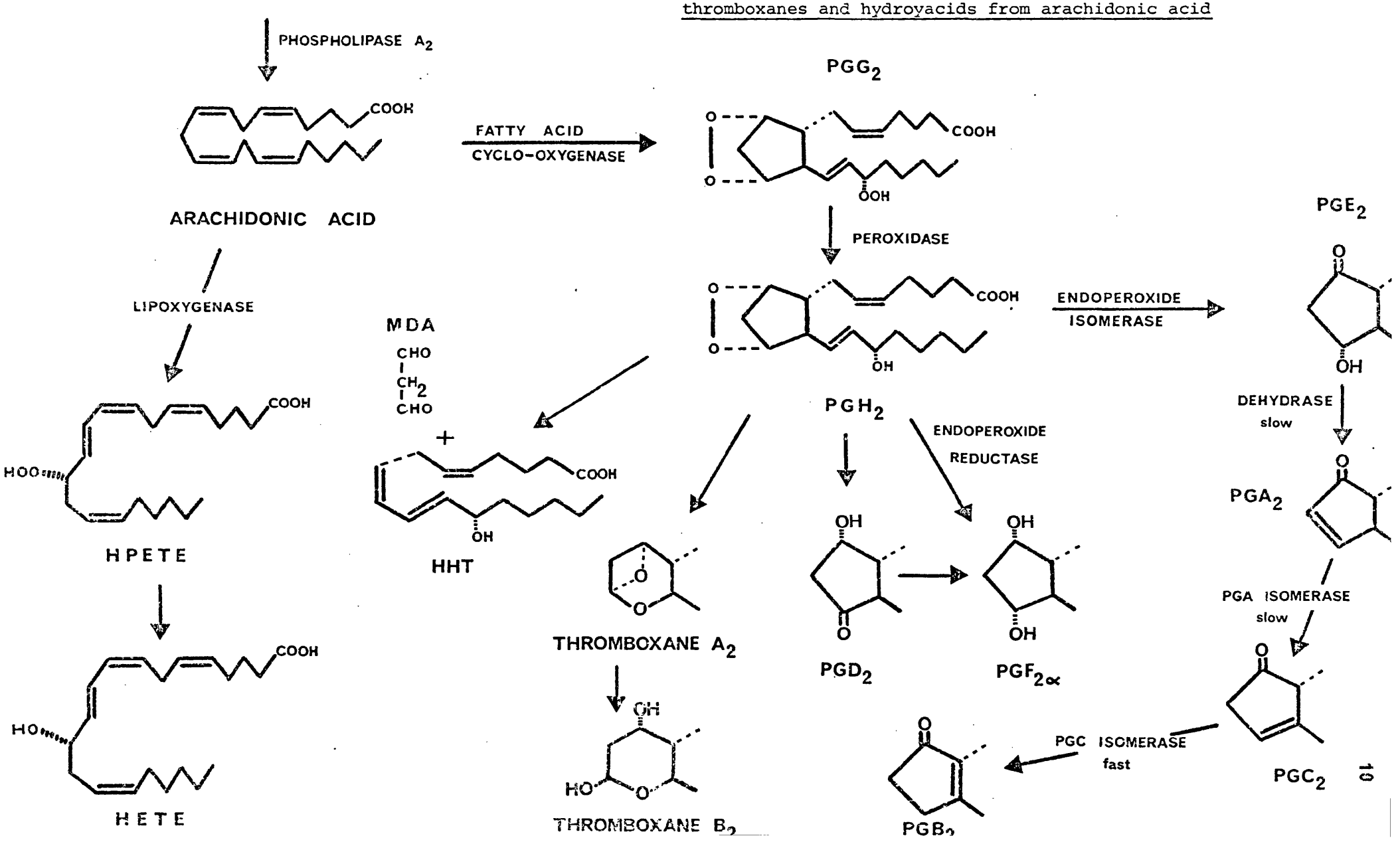
The initial step in the reaction is the stereospecific removal of the hydrogen atom at C-13, followed by isomerisation of the 11-cis double bond to 12-trans with the simultaneous attachment of molecular oxygen at C-11. A concerted reaction then follows, with the addition of molecular oxygen at C-15, ring closure between C-8 and C-12, and formation of a "cyclic endoperoxide" between C-9 and C-11. In agreement with this mechanism, 2 moles of oxygen are consumed for each mole of product formed, and elegant studies using $^{16}O_2$ - $^{18}O_2$ mixtures have shown that the oxygen atoms at C-9 and C-11 in prostaglandins derive from the same oxygen molecule (Ryhage and Samuelsson, 1965). The mechanism thus explains why only prostaglandins with a cis relationship between the hydroxyl groups at C-9 and C-11 are naturally occuring i.e. PGF_{α} and not PGF_{β}. Studies by Wlodawer and Samuelsson (1973) of the action of PG synthetase from sheep seminal vesicle microsomes on octa-deutero arachidonic acid also indicate that a common synthetase provides the endoperoxide precursor for both PGE_2 and $PGF_{2\alpha}$.

The observation that prostaglandin biosynthesis in bovine seminal vesicle microsomes is inhibited by catalase suggests that synthesis proceeds via a free-radical mechanism and that hydrogen peroxide may play a role as the source of the initial radical, possibly a hydroxyl radical. (Panganamala, 1974). It is well known that compounds such as hydroquinone stimulate PG biosynthesis and these compounds generate hydrogen peroxide through a quinone-hydroquinone cycle (Deamer and others, 1971). Furthermore, the possible participation of an Fe^{++}-enzyme complex in the decomposition of hydrogen peroxide to yield hydroxyl radicals may explain the observed enhancement of PG biosynthesis by some heme compounds (Yoshimoto, Ito and Tomita, 1970).

The cyclic endoperoxide PGH is the key intermediate in prostaglandin biosynthesis, and may be modified in a number of ways by the 'isomerase' component(s) of the enzyme complex. Isomerisation may yield PGE or PGD, and reductive cleavage leads to the formation of PGF_{α}. Cleavage of carbon-carbon bonds may also occur to yield a C_{17} hydroxyacid (12-hydroxy-5, 8, 10-heptadecatrienoic acid, HHT) and a C_3 fragment (malondialdehyde). Recent studies (Hamberg, Svensson and Samuelsson, 1975; Needleman and others, 1976) have identified an enzyme in platelet microsomes which is able to convert the cyclic endoperoxides PGG_2 or PGH_2 into an extremely unstable ($t_{\frac{1}{2}}$ at 37°C approx. 30 sec.) and biologically active non-prostanoid, oxane compound named thromboxane A_2 (TXA_2). This highly active compound decomposes to the stable and biologically inactive thromboxane B_2 (TXB_2, formerly named PHD). Finally, polyunsaturated fatty acids such as arachidonic acid may be metabolised by enzyme systems other than the well-documented fatty acid cyclo-oxygenase responsible for prostaglandin and thromboxane biosynthesis. Lipoxygenases may convert arachidonic acid to compounds such as 12-hydroperoxy-5, 8, 10, 14-eicosatetraenoic acid (HPETE) and 12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (HETE) though these are only produced in small amounts and their biological properties have not been extensively investigated.

Key stages in the biosynthesis of prostaglandins, thromboxanes and certain hydroxy acids are shown in Fig 1.2. The E and F series are the primary products of prostaglandin biosynthesis (Hinman, 1972). PGA and PGB are dehydration products of PGE and may be easily formed from PGE by treatment with acid or base respectively. A PGA isomerase has been demonstrated in rabbit plasma (Jones, 1974) but there has been no conclusive evidence for a 'PGE-dehydratase' which could convert PGE to PGA. It is difficult, however, to envisage a mechanism by which

Fig. 1.2 Pathways for the biosynthesis of prostaglandins, thromboxanes and hydroyacids from arachidonic acid



PGA could be biosynthesised other than through PGE. This is of interest to the renal physiologist as the occurrence of PGA_2 in renal tissue and its actions on renal and cardiovascular tissue have provided evidence for its involvement in the physiology of natriuresis and blood pressure regulation (Lee, 1973). However, doubt has recently been cast on the significance of PGA_2 in renal haemostasis (Frölich and others, 1975). Using an elegant GC/MS technique these authors showed that rabbit renal medulla homogenates contained no detectable PGA_2 and were incapable of biosynthesising it in vitro, raising the somewhat disturbing possibility that PGA_2 found in renal tissue by other workers may have been an artefact formed from PGE_2 during extraction and isolation procedures.

The conversion of PGE to PGF has been demonstrated using the cytoplasmic fraction from guinea pig liver (Hamberg and Israelsson 1970) i.e. PG-9-ketoreductase activity, and this can also be brought about by an enzyme from sheep blood (Hensby, 1974a). The significance of these observations at the present time is not known. A similar conversion of PGD_2 to $PGF_{2\alpha}$ i.e. PG-11-ketoreductase activity, has also been reported (Hensby, 1974b). Conversion of $PGF_{2\alpha}$ to PGE_2 i.e. PG-9-hydroxydehydrogenase activity, has been reported in rat kidney (Pace-asciak, 1975).

Cofactor requirements

Although PG synthetase activity is found in the microsomal fraction of cells, heat-stable cofactors from the cytoplasmic fraction are necessary for appreciable activity to be demonstrated in vitro. As the cytoplasmic fraction also contains prostaglandin metabolising enzymes, it is usual to supply these cofactors in the form of reduced glutathione (GSH) and a phenolic compound such as hydroquinone (HQ) or adrenaline, which act as a supply of reducing equivalents to recycle the GSH in its reduced form. The exact role of GSH is not known,

though its function may be a specific one as several other thiol compounds are inactive in this respect (van Dorp, 1967). Lands, Lee and Smith (1971) have postulated that GSH stimulates the internal 1,2-hydride shift in the cyclic endoperoxide intermediate and hence explains the commonly observed enhancement of PGE production by GSH at the expense of PGF. Cupric ions and dihydrolipoamide acted in the opposite way (Lee and Lands, 1972), and the role of copper in prostaglandin biosynthesis will be fully discussed later (section 4.4). These results thus lend further support to the conclusion that the cyclic endoperoxide is a common precursor of both PGE and PGF.

Yoshimoto, Ito and Tomita (1970) showed that in addition to GSH and HQ, the synthetase system from bovine seminal vesicles was stimulated by haemoglobin, myoglobin or haemin and concluded that haem compounds and HQ were involved in the step in which molecular oxygen became attached to the substrate. GSH, however, was involved in the reduction of the cyclic endoperoxide, as shown by the close relationship between PGE_2 formation and GSH utilisation. Whether or not this reduction is catalysed by glutathione peroxidase remains unanswered.

L-tryptophan may act as a cofactor for the biosynthesis of the cyclic endoperoxide (Miyamoto, Yamamoto and Haishi, 1974), but is unable to convert PGG or PGH to PGE or PGF. It is interesting to note that substantial quantities of L-tryptophan may be isolated from the supernatant fraction of bovine vesicular gland homogenates (Chan and others, 1975), raising the possibility that L-tryptophan may be the natural cofactor for the prostaglandin endoperoxide synthetase component, at least in bovine vesicular gland.

Substrate specificity

Only fatty acids containing at least three conjugated cis

double bonds in the 6, 9 and 12 positions from the terminal methyl group are substrates for PG synthetase. Of these, good conversion is only achieved with a chain length of 19, 20 or 21 carbon atoms (van Dorp, 1967). Furthermore a free carboxyl group is essential for conversion to occur, the corresponding fatty acid methyl esters being very poor substrates (Struijk and others, 1966). Similarly poly-unsaturated alcohols such as dihomo-γ-linolenyl alcohol and arachidonyl alcohol are not converted to the corresponding (hypothetical) prostanols (van Dorp, 1967).

Lands and Samuelsson (1968) showed that 1-palmitoyl-2-/ 2' -^{14}C / eicosatrienoyl glycero-3-phosphoryl choline was not a substrate for PG synthetase and that the ^{14}C-eicosatrienoic acid had to be cleaved from the phospholipid before conversion to ^{14}C-PGE_1 could occur. Similarly, Venkeman and van Dorp (1968) showed that incubation of sheep seminal vesicle microsomes with 1-stearoyl-2-/ ^{3}H / arachidonyl-lecithin produced no ^{3}H-PGE_2 and that prostaglandin synthesis took place only after addition of lecithinase A to the incubate.

Control of PG biosynthesis

The above findings are of prime importance to an understanding of the factors controlling prostaglandin biosynthesis and release as, with the exception of seminal fluid in man and some animals, prostaglandins do not exist in the body as pre-formed 'stores' but are rapidly synthesised and released in situ in response to a variety of neuronal, hormonal or even mechanical stimuli. In tissues processed to prevent biosynthesis during homogenisation and extraction, prostaglandin levels are virtually undetectable (Jouvenaz and others, 1970) and reports in the early literature of prostaglandin levels in tissues are probably only a measure of biosynthetic potential.

In most tissues, levels of free i.e. non-esterified, polyunsaturated fatty acids are extremely low; for example seminal vesicles contain only 10-50ng arachidonic acid per gram of wet weight tissue. As most tissues are able to rapidly produce amounts of prostaglandins which are orders of magnitude greater than this level of free substrate, in response to suitable stimuli, it is clear that a control mechanism must exist in cells which is capable of making appropriately high concentrations of substrate available for PG biosynthesis.

In most tissues, the polyunsaturated fatty acids form part of the phospholipid membrane structure and thus constitute a ubiquitous source of substrate for an apparently ubiquitous enzyme. Samuelsson (1972) has postulated that the hydrolytic release of the precursor acids, catalysed by phospholipase A, could be the rate-limiting step in the production of prostaglandins, and that the control of prostaglandins by humoral and neural mechanisms could be exerted at this site.

Recently, Flower and Blackwell (1976) have shown directly that phospholipase A_2 is indeed an important regulatory enzyme for the biosynthesis of prostaglandins. Slices of guinea pig spleen were incubated in buffer containing $/\overline{1\text{-}^{14}C}/$ arachidonic acid, the label being partially incorporated into cellular phospholipid and neutral lipid pools. When the tissues were mechanically vibrated, or subjected to anaphylactic shock, there was a significant release of labelled arachidonic acid from the phospholipid pools only, and an appropriate increase in the synthesis of labelled PGE_2. The release of arachidonic acid from the phospholipid pool was blocked by mepacrine, a known phospholipase inhibitor, though the effect on subsequent PG synthesis was difficult to estimate as mepacrine is an inhibitor of PG synthetase at the concentrations used to block phospholipase activity ($10^{-3}M$).

These results thus confirm that phospholipase A_2 is a key enzyme involved in the release of non-esterified fatty acid from phospholipid precursors prior to PG biosynthesis. However, it was also found that when spleen slices were vibrated in the presence of labelled arachidonic acid without prior incorporation into phospholipid pools, PG synthesis was increased several-fold over control (i.e. non-vibrated) incubations. This suggests that damage to the cell membrane may change its permeability in such a way as to allow increased access of non-esterified substrate to the PG synthetase 'compartment', and hence increased PG synthesis. Availability of substrate, therefore, may not be the only factor involved in the control of PG production.

1.4 Prostaglandin metabolism

Most prostaglandins are rapidly metabolised when introduced into the circulation or when incubated with broken-cell homogenates in vitro. Metabolism is initiated by oxidation at C-15 to form a 15-oxo derivative, the enzyme responsible being prostaglandin 15-hydroxydehydrogenase (PGDH). Reduction of the Δ^{13} double bond by prostaglandin reductase is followed by both β and ω oxidation.

The metabolising enzyme which has been studied in most detail is PGDH; it is NAD dependent and is found in the cytoplasm of cells, as are the other metabolising enzymes. The distribution of PGDH was studied in swine tissues by Anggard, Larsen and Samuelsson (1971) who found the highest concentrations in lung, spleen and renal cortex; smaller amounts were present in testicle, stomach, small intestine, heart and adipose tissue. The enzyme is also found in most tissues of rat, guinea pig and man, a particularly high level of activity being present in human placenta (Anggard and Samuelsson, 1966). PGE_1 and PGE_2 are the best substrates for PGDH; F and A-type prostaglandins are less readily oxidised and B prostaglandins are not oxidised at all.

Important species differences exist in the metabolism of administered prostaglandins. Thus ^{3}H-PGE_2 administered intravenously to human subjects is rapidly converted to 11α-hydroxy-9,15-diketo-prost-5-enoic acid (by oxidation of C-15 hydroxyl and reduction of Δ^{13}). Further degradation yields several metabolites which are mainly excreted in the urine, the principal one being 7α-hydroxy-5,11-diketo-tetranor-prostane-1,16-dioic acid (Hamberg and Samuelsson, 1969). $PGF_1\alpha$ and $PGF_2\alpha$ are metabolised to the corresponding 3α, 7α-dihydroxy-11-keto-tetranor-prostane-1,16-dioic acid (Granström and Samuelsson, 1971). In the guinea pig, however, the major urinary metabolite of ^{3}H-PGE_2, given intravenously is 5β, 7α-dihydroxy-11-keto-tetranor-prostanoic acid (Hamberg and Samuelsson, 1969), and in the rat administration of ^{3}H-$PGF_1\alpha$ produces dinor-$PGF_1\alpha$ as the major urinary metabolite (Granström, Inger and Samuelsson, 1965).

A systematic study of PG metabolism in lung tissue homogenates was made by Anggard and Samuelsson (1964; 1966) using both guinea pig and pig lung. Incubation of PGE_1, PGE_2 or PGE_3 with a homogenate of guinea pig lung produced a mixture of the corresponding 13,14-dihydro and 13,14-dihydro-15-oxo prostaglandins whereas pig lung homogenates incubated with PGE_1 produced only the 15-oxo derivative. Incubation of ^{3}H-PGE_1 with the β-oxidation system of rat liver mitochondria showed PGE_1 to be converted to dinor-PGE_1; 13,14-dihydro-PGE_1 produced both dinor and tetranor metabolites and $PGF_2\alpha$ was transformed into a C_{16} derivative. Studies with the microsomal fraction of human liver (Hamberg and Samuelsson, 1968) demonstrated the transformation of PGA_1, but not PGE_1, into the 19-hydroxy prostaglandin. This is consistent with the presence of 19-hydroxy-PGA_1 in human seminal plasma.

1.5 General pharmacology

The original findings of large amounts of prostaglandins in

the male accessory genital glands and their secretions, and subsequent discovery in menstrual and amniotic fluids showed that these substances were intimately linked with several aspects of reproduction. The recent avalanche of research into all aspects of prostaglandin pharmacology and physiology which has followed these early observations has provided much evidence for the involvement of prostaglandins in the physiology of many bodily functions, and the pathological disorders of these functions. Disturbed prostaglandin production might contribute to the symptomology associated with some pathological conditions, and administration of prostaglandins or inhibitors of prostaglandin synthesis or action may limit the symptoms. This is of particular interest in the iatrogenic pathology associated with the ingestion of many of the commonly used anti-inflammatory drugs (see section 1.12).

The pharmacological effect of prostaglandins on the respiratory system has been studied as the lungs are the principal site of metabolism of circulating prostaglandins in the body, both inactive and biologically active metabolites being formed. Interest has centred around the possible involvement of prostaglandins in the pathogenesis of asthma. Prostaglandins of the E series will inhibit, and prostaglandins of the F series will increase bronchial tone in man (Smith and Cuthbert, 1973) leading to speculation of a possible imbalance between PGE and PGF production in asthmatic patients. Furthermore, such patients show increased airway resistance when they inhale an aerosol of $PGF_2\alpha$, in quantities thousands of times smaller than those producing a comparable effect in normal subjects (Smith, 1972). The bronchodilator effect of E prostaglandins in man suggests a possible use of these compounds in the treatment of asthmatic symptoms.

Prostaglandins also have varied actions on the cardiovascular system. Thus, E and A series prostaglandins decrease total peripheral

resistance whereas the F prostaglandins are vasoconstrictor. PGE_2 possesses antiarrhythmic activity in experimental animal models and also increases coronary blood flow (Wennmalm and Hedquvist, 1970). The effect of prostaglandins upon regional blood flow can be variable and important species differences exist.

The occurrence of prostaglandins in renal tissue and their actions on renal and cardiovascular tissue have provided evidence for their involvement in the physiology of natriuresis and blood pressure regulation (Lee, 1973). In normal subjects infusion of PGE_2 or PGA_2 causes increased cortical blood flow and a concomitant decrease in medullary flow. Infusion in hypertensive patients results in a fall in blood pressure accompanied by natriuresis, diuresis and increased GFR.

Secretions and motility of the gastrointestinal tract can be radically modified by prostaglandins. Both E and F prostaglandins cause the longitudinal muscle of the gut to contract, but the circular layer is relaxed by PGE_2 and contracted by $PGF_{2\alpha}$. Prostaglandins are also potent inhibitors of gastric acid and pepsin secretion, the newer synthetic PG analogues being particularly active in this respect. Not surprisingly such compounds have undergone clinical trials for the treatment of gastric ulcers.

The role of prostaglandins in inflammation is discussed in greater detail in section 1.8 of this chapter. Similarly the role of prostaglandins, their cyclic endoperoxides and the newly discovered thromboxanes, in platelet aggregation is to be found in section 5.1.

B. INFLAMMATION, ASPIRIN-LIKE DRUGS AND PROSTAGLANDINS

1.6 The Inflammatory Process

The physiological and biochemical changes which occur during inflammation have been the subject of inumerable books, reviews and papers; notable amongst these is the 'standard' text of Zweifach, Grant and McCluskey (1974).

Inflammation has been defined by Ebert and Grant (1974) as 'a process which begins following sublethal injury to tissue and ends with permanent destruction of tissue or with complete healing'. Even such a general definition may fail to take account of the apparent 'stalemate' often seen clinically with the chronic inflammatory diseases such as rheumatoid arthritis. The four cardinal signs of inflammation, redness, swelling, heat and pain were described by Celcus (30B.C. - 38A.D.) and the fifth, loss of function, added by Galen (130-200A.D.) Regardless of the initial inflammatory stimulus or the location of the injured tissue the immediate effect is that cells are adversely affected by the insult and an inflammatory reaction is initiated.

This response is usually brought about by the release of intracellular contents into the extracellular space with a subsequent response in the surrounding capillaries. Vasoactive mediators such as histamine and 5-hydroxytryptamine are liberated, primarily from mast cells. These increase the permeability of the venules and capillaries to circulating macromolecules and cells, resulting in the accumulation at the injured site of fluid containing, initially, a predominantly polymorponuclear (PMN) cell population, due to the greater ease with which the PMN leucocyte is able to penetrate the vascular endothelium (Paz and Spector, 1962).

The accumulation of leucocytes at an inflammatory focus may be explained by the phenomenon of chemotaxis, i.e. the liberation or formation at the inflammatory site of substances which produce an attraction for leucocytes which over-rides their normal random migration. Numerous such chemotactic attractants have been demonstrated in vitro, notably the production of chemotactic factors from complement activation (Cochrane, 1968). Products of the inflammatory reaction may themselves account for cell specific chemotaxis, as kallikrein formation, due to activation of the kinin system, releases chemotactic factors specific for neutrophils (Kaplan, Kay and Austen, 1972). Other mediators such as cyclic nucleotides and prostaglandins are also released, their relatively short action being terminated either by catabolism or dilution within the extracellular space. Synthesis and/or release of these mediators may occur either from the locally damaged tissue, or from the invading leucocytes; thus histamine is transported by the basophil and eosinophil (Graham and others, 1955), 5-hydroxytryptamine by the platelet (Humphrey and Jacques, 1954), while prostaglandins are synthesised and released by platelets (Smith and Willis, 1971) and PMN's (Higgs and Youlten, 1972).

Fibrin deposition and platelet aggregation occur late in the inflammatory response causing micro-thrombi formation. Prostaglandins were shown to be only weakly pro-aggregatory or even, in the case of PGE_1, an inhibitor of platelet aggregation (Kloeze, 1967). Recently however, platelets have been shown to be capable of producing prostaglandin endoperoxides and thromboxanes of the '2' series possessing potent pro-aggregatory properties (Hamberg, Svensson and Samuelsson, 1975). Such localised thrombi formation may be important, resulting in stasis, ischaemia and anoxia, leading

to possible tissue necrosis. As discussed later (section 1.11) the aspirin-like drugs have been shown to be potent inhibitors of prostaglandin production.

Inflammatory exudates also contain a number of proteases, esterases, cathepsins and lytic enzymes, including collagenases and nucleases, capable of degrading protein-polysaccharide complexes within surrounding connective tissue. These enzymes are derived from lysosomal granules of the invading PMN's or from phagocytic cells within the tissue. Inevitable destruction of the epithelial basement membrane by these enzymes facilitates the entry of further leucocytes. The final cell scavengers in an acute inflammatory reaction are the monocytes, which phagocytose any remaining debris before returning to the circulation. This is in contrast to the typical chronic inflammation exemplified by rheumatoid arthritis, where there is colonization of the inflammatory focus by the monocytes.

One of the most important, though least well understood aspects of inflammation is the transition from the initial beneficial, acute inflammation to a degenerative, chronic inflammation. It is now thought that the chronic response is a result of the generation and release of 'autoantigens' from the injured tissue, possibly involving a modification of normal tissue components by lysosomal enzymes (Weissman, 1964).

The inflammatory stimulus is thus self-perpetuating and able to continue the process long after the original initiator has been removed. Such an explanation would account for the fact that experiments designed to isolate and identify the antigen responsible for initiating the events leading to chronic degenerative inflammation in man have been uniformly unsuccessful.

Investigations into the mode of action of a drug in a pathological process need to be based on an intimate knowledge of the physiological and biochemical changes which have occurred in the tissue and which the drug is, hopefully, to reverse or correct. It is possible that an understanding of the mode of action of therapeutically useful drugs might also lead to the rational design of more effective drugs, and provide some insight into the pathogenesis of the disease itself.

1.7 Rheumatoid Arthritis (R.A.)

Rheumatoid arthritis is a chronic, systemic, degenerative disease, principally affecting the distal joints of the limbs. The disease is found only in man, its etiology is unknown and there is no known cure.

The joint inflammation is characterised by a proliferating, hyperaemic synovium with cellular infiltration, and exudation into the synovial space. Lymphocytes are the predominant cell type found within the synovium and have a tendency to aggregate around small blood vessels. That the lymphocyte plays a key role in the pathogenesis of R.A. is shown by the finding that prolonged thoracic-duct drainage, leading to lymphocyte depletion, ameliorates the activity of the disease (Wegelius and others, 1970). Much evidence now exists suggesting that cell-mediated immunity may play a role in the pathogenesis of R.A. This is beyond the scope of the present brief description of the disease and is comprehensively reviewed by Glynn (1972), Barland (1973) and Yu and Peter (1974).

As discussed above, a critical event, or series of events in the development of the disease is the transition from an initial reversible synovitis to one in which the synovium hypertrophies to

form a granulocytic tissue called "pannus". Cartilage is undermined and eroded at its margins, leading to erosion and digestion of the underlying bone matrix, with the pannus occupying the space normally taken by cartilage and thus preventing re-growth of normal cartilage. This erosion is thought to be due to proteolytic and other enzymes released in the main from lysosomes of the infiltrating cells; the presence of such enzymes may be readily demonstrated in both rheumatoid synovium and in synovial fluid (Hamerman, Stephens and Barland, 1961). Though most of the common tissue proteases have optimum activity at acid pH's unlikely to be found in vivo, it has been shown that collagenase is present in inflamed synovial tissue (Evanson, Jeffrey and Krane, 1968), an enzyme capable of digesting native collagen at or near to neutral pH.

Most R.A. synovial fluids possess considerable chemotactic activity for leucocytes in vitro, and this activity may be due to products of the complement system (Cochrane, 1968), fibrin and its degradation products (Riddle, Bluhm and Barnhart, 1965), kinins (Melmon and others, 1967) and prostaglandins (Higgs, McCall and Youlten, 1975).

Prostaglandins are found in raised levels in rheumatoid synovial fluids (Levine, 1973; Higgs and others, 1974) though the levels do not correlate with either the clinical index of inflammation in the joint or the leucocyte count in the fluid (Swinson, Bennett and Hamilton, 1976). It is not possible however to conclude from these results either that prostaglandins do not contribute significantly to the inflammation or that leucocytes are not the source of synovial fluid prostaglandins, as prostaglandins may be rapidly cleared from the joint (Ferreira, 1976). The role of prostaglandins in inflammation and their possible involvement in R.A.

are discussed in detail in the following two sections.

1.8 The role of prostaglandins in inflammation

Prostaglandins are generated in many forms of tissue damage in both animals and man including carrageenin inflammation (Willis, 1969), thermal injury to the skin (Jonsson, 1971), anaphylaxis (Piper and Vane, 1969), monoarticular arthritis (Blackham and others, 1973), experimental uveitis (Eakins and others, 1973), allergic contact eczema (Greaves, Søndergaard and McDonald-Gibson, 1971), ultraviolet-induced inflammation (Greaves and Søndergaard, 1970) and rheumatoid arthritis (Levine, 1973; Higgs and others, 1974).

Much evidence now exists which shows the prostaglandins to be capable of either producing the classical signs and symptoms of inflammation, or of augmenting the inflammatory effects of other mediators such as histamine, 5-hydroxytryptamine and bradykinin. Current thinking places more emphasis on the latter possibility, namely that prostaglandins act in many inflammatory processes as modulators rather than mediators of inflammation. Paradoxically it is also possible to demonstrate some anti-inflammatory properties of certain prostaglandins, leading to speculation that the involvement of prostaglandins at an inflammatory focus may be a function of a qualitative rather than a quantitative change in prostaglandin production (Stone, Mather and Gibson, 1975).

In different types of inflammation some mediators may have a more prominent role than others and the involvement of each one may follow a very different time course in terms of release and maintenance of effect. It would thus be a gross oversight to focus ones attention solely on the prostaglandins as mediators or modu-

lators of inflammation without taking into account the participation of the other mediators involved. Recognition of this fact may go some way towards explaining the inability of the aspirin-like drugs to arrest the disease process in a chronic condition such as rheumatoid arthritis, and has given rise to suggestions that the ideal anti-inflammatory therapy might consist of a 'cocktail' mixture of drugs with each component directed at one particular mediator or modulator of the inflammation.

(a) Inflammatory properties of prostaglandins

(i) Erythema

Prostaglandins of the E and F series cause erythema in man and animals, the E series being considerably more potent than the F series. Bergström and others (1959) demonstrated a dull erythema after the infusion of PGE_1 into the forearm and Ambache (1962) showed a similar effect with an extact of iris ('irin'), later shown to contain high concentrations of PGE_2 and $PGF_{2\alpha}$. Notable features of the vascular effects of prostaglandins are the long-lasting action in producing erythema and the ability to counteract the vasoconstriction caused by such substances as noradrenaline and angiotensin. It should be noted however that the long-lasting effect applies only to cutaneous vessels and superficial veins, the vasodilator effect on other vascular beds vanishing within a few minutes. The long-lasting erythema induced by PGE_1, measured thermographically, is also seen in rat paw (Åberg, 1973).

(ii) Oedema

In the rat and in man prostaglandins cause increased vascular permeability by inducing leakage at the postcapillary and collecting venules (Kaley and Weiner, 1971). The vascular permea-

bility of guinea pig skin is little affected by the application of prostaglandins alone, but they cause a marked potentiation of the response to either histamine or bradykinin (Williams and Morley, 1973). Prostaglandins E_1, E_2 and A_2, though not $F_2\alpha$, cause oedema when injected into rat paws (Glenn, Bowman and Rohloff, 1972) though the effect is not dose-related as is the case with bradykinin. The use of PGE_1 together with either histamine or bradykinin showed a synergistic potentiation of the oedema response rather than a simple additive effect, though there was no addition of effect between histamine and bradykinin (Moncada, Ferreira and Vane, 1973). Lewis, Nelson and Sugrue, (1974) have shown a potentiated oedema response to carrageenin and kaolin by arachidonic acid.

Thomas and West (1973) made the interesting observation that whilst PGE_1 selectively potentiated the increased permeability induced in rat skin by bradykinin, PGE_2 was without effect and $PGF_2\alpha$ actually inhibited the effects of other mediators. PGE_1 however had no effect on dextran-induced oedema. The inhibitory effect of $PGF_2\alpha$ may be peculiar to rat skin as bradykinin-induced oedema in the rabbit is not inhibited by $PGF_2\alpha$.

In general the oedema-inducing effects of the prostaglandins are not as marked as their erythema-inducing, i.e. vasodilator, effects and the effect is not long-lasting compared with the erythema.

(iii) Pain

In man high doses of prostaglandins administered intradermally or intramuscularly cause long-lasting overt pain, and intravenous prostaglandins cause headache and pain along the veins into which they are infused (Bergström and others, 1959). However, in 'realistic' concentrations likely to be found at inflamed sites

prostaglandins induce a state of hyperalgesia i.e. a state of hypersensitivity to normally painless stimuli. This sensitising action at the pain receptors is cumulative and long lasting and has been observed in man when PGE_1 was given intradermally or infused subdermally (Ferreira, 1972). The observation has been confirmed in dog spleen, dog knee joint and rat paw. As with the oedema response, the effect of PGE_1 was to potentiate the pain-producing effects of bradykinin and/or histamine, as separate infusions of PGE_1, bradykinin or histamine produced no overt pain. Similarly, none of these three substances cause pruritus when infused separately, but when PGE_1 was infused together with histamine, itch was always recorded.

Prostaglandins applied topically to a blister base do not cause pain, possibly because the site is already 'saturated' with prostaglandins (Horton, 1963).

A lipoxygenase system is present in many tissues together with the prostaglandin forming cyclo-oxygenase system, and the fatty acid hydroperoxides generated by the action of lipoxygenase on arachidonic, linoleic or linolenic acids, have been shown to cause pain in man when injected intradermally (Ferreira, 1972). Due consideration therefore must be given to the pharmacological effects of non-prostanoid products of fatty acid metabolism; in some tissues prostaglandins may represent only a small percentage of the total products of arachidonic acid metabolism (Hamberg and Samuelsson, 1974a, 1974b).

(iv) Fever

Prostaglandin E_1 is the most potent pyrogen known when injected into the third cerebral ventricle of rabbits, rats and cats or directly into the anterior hypothalamus (Milton and Wendlandt, 1971; Feldberg and Saxana, 1971). The effect is dose-dependent,

almost immediate and lasts for about three hours. During fever there is an increase in the level of a prostaglandin E-like substance in the central nervous system, and concentrations in the CSF rise severalfold after intravenous pyrogen (Feldberg and Gupta, 1973).

(v) Loss of function

Injections of PGE_1 and PGE_2 into the dog knee joint induce a long-lasting inflammatory effect, causing incapacitation within fifteen minutes; $PGF_{2\alpha}$ produces a more gradual incapacitation over several hours. (Rosenthale and others, 1972). As with oedema and pain the action of bradykinin in this model has been reported to be potentiated by prostaglandins (Ferreira, Moncada and Vane, 1973).

(vi) Leucocyte migration

Studies of the effect of prostaglandins on the migration of leucocytes, both in vitro and in vivo, have produced conflicting data. Kaley and Weiner (1971) reported PGE_1, but not A_1, E_2, or $F_{2\alpha}$, to be chemotactic for rabbit peritoneal polymorphonuclear leucocytes in vitro, though the concentration used was high (1µg/ml). Furthermore the PMN's were shown to be the main source of prostaglandins in immunogenic uveitis in rabbits (Eakins and others, 1972). More recently it has been reported that PGE_1 is chemotactic in concentrations as low as 10ng/ml i.e. a concentration less than that typically found in inflammatory exudates (Higgs, McCall and Youlten, 1975). It has thus been suggested that prostaglandins produced during phagocytic activity could act as chemotactic stimuli for further phagocyte infiltration of the inflamed area.

Other observations however cast doubt upon the importance of PGE_1 as a leucotactic agent. Thus intradermal injection of PGE_1 in the skin of both rat and man failed to cause increased leucocyte

migration (Arora, Lahiri and Sanyal, 1970). Another finding of great interest is that freshly prepared solutions of PGE_1 do not exhibit chemotactic activity against either human or rat peripheral leucocytes *in vitro*, even at a concentration of 1µg/ml (Turner, Campbell and Lynn, 1975; Ford-Hutchinson, Smith and Walker, 1976), though such activity can be detected in aged solutions. The latter authors have recently shown that although PGE_1 does exhibit chemotactic activity against rabbit peritoneal PMN's it is devoid of such activity against similar cells obtained from the rat or against peripheral PMN's from rabbit, rat or man (Walker, Smith and Ford-Hutchinson, 1976).

(vii) Granuloma formation

An important aspect of the inflammatory reaction is the granuloma formation associated with increased collagen production. PGE_1 enhances the granuloma formation by implanted cotton pellets (Arora, Lahiri and Sanyal, 1970) and increases collagen synthesis in check embryo tibia (Blumenkrantz and Søndergaard, 1972). However in rats PGE_1 did not elicit a granulomatous reaction when injected into air blebs at high doses (50-100 µg) (Glenn, Bohman and Rohloff, 1972). The administration of aspirin-like drugs diminished cotton pellet granuloma (Winter, Risley and Nuss, 1963).

(viii) Cartilage metabolism

Preliminary results have been presented which suggest that prostaglandins produced by rheumatoid synovial cultures reduced the incorporation of glycine, leucine and sulphate into normal rabbit articular cartilage *in vitro* and may therefore be inhibiting protein-polysaccharide synthesis (Robinson and McGuire, 1975). Another report has described inhibition of chondromucoprotein synthesis

of chicken and rat cartilage by high concentrations of PGA_1, though not by PGE_1 or PGE_2 (Eisenbarth, Beuttel and Lebovitz, 1974).

(ix) Bone resorption

One of the most interesting aspects of the role of prostaglandins in chronic inflammatory conditions such as rheumatoid arthritis is that they may be capable of promoting bone resorption and erosion, thus being fundamentally implicated in the destructive disease process as opposed to merely contributing to the signs and symptoms of the inflammation. Klein and Raisz (1970) reported that PGE_1 and PGE_2 caused resorption of foetal rat bone in vitro and Tashjian and others (1972) showed that the ability of a mouse fibrosarcoma to cause hypercalcaemia in mice and promote bone resorption in mouse calvaria organ cultures was attributable to the production of large amounts of PGE_2 by the tumour cells. Robinson, Tashjian and Levine (1975) have implicated PGE_2 directly in the pathogenesis of rheumatoid arthritis by demonstrating a high level of bone resorption-stimulating activity in culture media from fragments of rheumatoid synovia, and have presented convincing evidence that this activity is due to the production of PGE_2 by the culture.

(b) Anti-inflammatory properties of prostaglandins

In spite of all the inflammatory properties of the prostaglandins described above, several reports have shown them to exhibit certain anti-inflammatory and anti-arthritic properties. (Zurier and Quagliata, 1971; Glenn and Rohloff, 1972). In general the E series are the most potent in suppressing various models of inflammation, though anti-inflammatory activity has been demonstrated for $PGF_{2\alpha}$ in both carageenin oedema and adjuvant arthritis (Dipasquale and others, 1973). It must be emphasised however that very large doses of prostaglandins are necessary to demonstrate these effects,

and that such doses may be producing pronounced pharmacological effects not found with physiological concentrations of prostaglandins.

Kalinder and Austen (1974) have postulated that the anti-inflammatory effects of prostaglandins may be mediated via the nucleotide levels of target cells. Treatment of phagocytosing PMN's with PGE_1 brought about a rise in cellular cyclic 3', 5'-adenosine monophosphate (cAMP) and a corresponding fall in the release of lysosomal enzymes; $PGF_{2\alpha}$ however produced the reverse effect. E series prostaglandins suppress antigen-induced release of histamine and slow reacing substance of anaphylaxis from human lung tissue (Tauber and others, 1973) and allergic histamine release from human leucocytes (Bourne, Lichtenstein and Melmon, 1972). Suppression of mediator release is associated with an increase in cellular cAMP, factors which decrease cAMP levels, such as phosphodiesterase activity, leading to greater mediator release. A finding of great interest is that although $PGF_{2\alpha}$ has little effect on cAMP levels it is capable of elevating cyclic guanosine monophosphate (cGMP) levels. These two cyclic nucleotides usually have opposing roles in controlling cellular functions (Goldberg and others, 1973), so it is possible that the E and F series prostaglandins oppose each others actions by altering cellular levels of the two cyclic nucleotides.

1.9 Prostaglandins in rheumatoid arthritis

Using a radioimmunoassay technique to measure PGB levels in human synovial fluids, Levine (1973) showed that in a group of patients with inflammatory effusions (arbitrarily defined as those containing more than 10^3 PMN's per cubuc millimetre) the mean concentration was significantly higher than that in a group with non-inflammatory

lesions. A group with inflammatory lesions who had received salicylate or indomethacin therapy, however, had the same mean PGB level as the non-inflammatory group. A similar study by Higgs and others (1974), using a bioassay technique to measure prostaglandins in R.A. synovial fluid, showed the average concentration in fluids from untreated patients to be seven times higher than that in fluids from patients receiving aspirin-like drugs. The hypothesis that aspirin-like drugs inhibit prostaglandin biosynthesis is fully discussed in section 1.11.

Studies of rheumatoid synovial fragments in culture have shown the tissue to be capable of producing both PGE_2 and $PGF_2\alpha$; the addition of indomethacin (5µg/ml) reduced prostaglandin levels to less than 2% of those found in both control and colchicine-stimulated cultures (Robinson and McGuire, 1975).

Synovial cultures from normal joints or from patients with degenerative or traumatic arthritis produced less PGE_2 than was found with active rheumatoid specimens. Furthermore an area of a rheumatoid synovium with active synovitis (as shown histologically) produced greater concentrations of PGE_2 and $PGF_2\alpha$ than did an area with less active inflammation, as compared on the basis of equal wet weights.

The cell(s) in which prostaglandin synthesis originates in rheumatoid synovium are unknown; however, fibroblast cell lines cultured from rheumatoid synovia retain the ability to produce large amounts of PGE_2. A comparison of synovial and skin fibroblasts showed that, in general, rheumatoid synovial cells produce higher levels of prostaglandins and cAMP than do normal skin cells from the same patients (Robinson and McGuire, 1975).

One of the most exciting findings to emerge from these studies is that a high level of bone resorption stimulating activity is present in culture media from fragments of rheumatoid synovia and that this activity is due to production of PGE_2 by the tissue (Robinson, Tashjian and Levine, 1975). This is the first piece of evidence that prostaglandins may be actively involved in the disease process rather than merely contributing to the symptomology. This is supported by the finding that isolated adherent rheumatoid synovial cells in culture vessels produce large quantities of both collagenase and PGE_2 (Dayer and others, 1976).

1.10 Anti-rheumatic therapy

The large number of drugs available for the treatment of a chronic inflammatory condition such as rheumatoid arthritis testifies to their individual inadequacy, and few clinicians would champion any as the 'drug of choice'. No treatment to date has been shown to significantly alter the course of the disease process in rheumatoid arthritis (Famaey, Brooks and Dick, 1975) though clearly much human suffering is alleviated by the use of such therapy.

(a) Aspirin-like drugs

Salicylates

Salicylates have occupied a key position in the treatment of rheumatoid arthritis for over 100 years. The use of willow bark for the treatment of various agues, fevers and abscesses was first described in 1763. By 1876 an extract of willow bark, 'salicin', was being used for the treatment of acute rheumatism and was shown to contain salicylic acid as the active constituent. Acetylsalicylic acid, under the trade name 'Aspirin' was first produced by the Bayer Company of Germany in 1899.

Aspirin exhibits analgesic and antipyretic properties with small doses (600mg) though much higher doses (2-5g. per day) are necessary to produce anti-inflammatory effects. At this dose many patients are unable to tolerate the side effects of the drug, the principal ones being gastric irritation and CNS disturbances. Gastric problems have been minimised by the use of new pharmaceutical preparations of aspirin involving buffered, microencapsulated or esterified forms of the drug.

Pyrazoles

Phenazone and amidopyrine were two of the earliest drugs in this group to be used in the treatment of rheumatic diseases, though neither are prescribed now due to their toxicity and tendency to cause agranulocytosis.

Phenylbutazone, (Butazolidin,) is the most commonly used member of this group of compounds, possessing potent antirheumatic effects and also being a weak analgesic. It is long acting and slowly metabolised, being firmly bound to plasma proteins, mainly albumin and α-globulin. Toxic effects, usually dose-related, are common and include the expected gastro-intestinal irritation and potentially dangerous effects on the bone marrow.

Indomethacin

Indomethacin, (Indocid), was first synthesised in 1963 (Shen and others, 1963) and first reported as a useful drug in the treatment of R.A. two years later (Norcross, 1965). Numerous reports since have confirmed its potent anti-inflammatory and analgesic properties, both in man and in animal models. In man it has a short duration of action, roughly 4 to 12 hours depending on the dose given, being rapidly absorbed and quickly eliminated in the urine as the glucuronide.

Side effects include gastric irritation, cerebral sensations, skin rashes and occasional ocular complications.

Proprionic acid derivatives

This group of compounds includes naproxen, (Naprosyn), ibuprofen, (Brufen), fenoprofen, (Fenopron), ketoprofen, (Orudis; Alrheumat) and flurbiprofen (Froben). All have been shown to possess anti-inflammatory and analgesic properties and to share the common unwanted side effects of gastric irritation, CNS disturbances and skin rashes. Numerous clinical trials have been carried out to compare their relative efficacy in the treatment of R.A. and to assess their undesirable side effects, often with conflicting results. A recent trial advocates naproxen as the drug combining the greatest effectiveness with the lowest incidence of side-effects (Huskisson and others, 1976) though doubtless many dissenting views could be found in the literature.

Anthranilic acid derivatives

Mefenamic acid, (Ponstan) and flufenamic acid, (Arlef) have been shown to be effective in the treatment of R.A. (Barnado and others, 1966; Rajan and others, 1967). They share the side effects common to all the drugs described above, together with a tendency to cause diarrhoea, and are not widely used in clinical practice.

(b) Anti-inflammatory steroids

Great excitement was aroused by the finding that the adrenocortical hormone, cortisone, had considerable activity in the treatment of rheumatoid arthritis (Hench and others, 1949). Not surprisingly, at the doses used initially (300mg/day) the results obtained were dramatic, but this initial euphoria quickly gave way to disillusionment when the highly undesirable side effects of corticosteroid overdosage began to appear. These included fluid and

electrolyte disturbances, hyperglycaemia and glycosuria, osteoporosis, poor wound healing and increased susceptibility to infection - nothing less than an iatrogenic Cushing's syndrome. Patent synthetic analogues of cortisone, such as prednisolone were later produced which caused fewer disturbances of fluid and electrolyte balance, and these remain highly effective drugs in the treatment of the disease when used at conservative dose levels. They are especially useful when given as an intra-articular injection to control a sudden 'flare-up' in a joint, and new pharmaceutical preparations have been devised which aim to provide a sustained action at the inflammatory focus with minimum systemic effects due to leakage from the joint.

The efficacy of corticosteroid therapy in the treatment of R.A. gave rise to the hypothesis that the supply of cortisol to the nuclei of cells susceptible to, or involved in R.A. might be abnormal i.e. deficient (Bailey and West, 1967) though this has apparently not been pursued.

(c) Other antirheumatic drugs

Gold

Gold salts were first introduced into medicine because they were thought to be effective in tuberculous disease. On the assumption that R.A. might be a disease of infective origin gold was tried as a treatment and found to be effective, thereby introducing a valid therapy on what is now thought to be a false theoretical basis. Several clinical trials have testified to the effectiveness of gold injections in the treatment of R.A., though the active joint disease is not arrested. The toxic effects of chrysotherapy, such as leucopenia, are formidable; these effects are not dose-related, often arise without prior warning and can be fatal.

Gold is one of the major causes of reported deaths from drug therapy (Girdwood, 1974).

Penicillamine

Penicillamine (Distamine), a powerful chelating agent, was first used in 1956 for the treatment of Wilson's disease. More recently a Multicentre Trial Group (1973) suggested that although slow in onset, penicillamine may actually slow the progression of rheumatoid arthritis. The drug dissociates macromolecules, decreases collagen synthesis (Nimmi and Bavelta, 1965) and reduces the titre of rheumatoid factor in the serum of patients with R.A. (Dresner and Trombly, 1960). Serious side effects of penicillamine therapy include gastro-intestinal upsets, skin rashes, albuminuria and agranulocytosis. Its use is therefore limited to situations where careful patient monitoring and supervision is possible.

Immunosuppressive drugs

As the pathogenesis of R.A. is now thought to have an immunological basis it was a logical step to test the efficacy of the known immunosuppressive drugs in the treatment of the disease. Cyclophosphamide and azathioprine have both been reported to be effective (Fosdick, Parsons and Hill, 1969; Fricke and Deicher, 1969) though fearsome side effects, including leucopenia, myelosuppression, hepatotoxicity and nephrotoxicity place severe limitations upon their usage in all but the most intractable of cases.

1.11 Inhibition of PG biosynthesis - the Vane hypothesis

The elucidation of the mode of action of the aspirin-like drugs, whereby they exert their anti-inflammatory, anti-pyretic and analgesic properties has been a topic of intense pharmacological interest for many years. Clearly the understanding of the mode of action of a

group of compounds widely used therapeutically is a highly desirable goal if future, more effective and less toxic drugs are to be designed on a rational basis. Furthermore, an understanding of the way in which a drug influences a pathological process may shed some light on the aetiology and progress of the disease itself, and possibly suggest new approaches to its treatment or even prevention.

Many hypotheses have been advanced to explain the pharmacological properties of the aspirin-like drugs; these have included uncoupling of oxidative phosphorylation (Adams and Cobb, 1958), inhibition of leucocyte phagocytosis (Chang, 1972), stabilisation of lysosomal membranes (Weissmann, 1972), displacement of anti-inflammatory peptides from serum proteins (Smith, Dawkins and McArthur, 1971) or interference with migration of leucocytes (Di Rosa, Giroud and Willoughby, 1971). These and other possibilities are presented in an excellent review by Famey, Brooks and Dick (1975) and will not be discussed here in detail. Suffice it to say that although each particular hypothesis has had its ardent supporters, all have been subject to telling criticism and none has achieved wide support. The most common objection raised to all of these hypotheses has been that very high concentrations of aspirin-like drugs are necessary to demonstrate the appropriate anti-inflammatory properties in vitro, concentrations usually far in excess of those which are achieved therapeutically in vivo. Aspirin-like drugs at high concentrations also inhibit protein biosynthesis and many cellular enzymes, but these effects are more likely to be observed in cases of drug overdose and intoxication than in normal therapeutic usage. A further objection to some of the hypotheses has been that no correlation can be demonstrated between the proposed mode of action of the aspirin-like drugs in vitro and their known anti-inflammatory

potency both in animal models and in the treatment of disease in man - with the lysosome theory, for example, there is an inverse correlation between the potency of the aspirin-like drugs as anti-inflammatory agents and their ability to stabilise lysosomal membranes.

In 1971, Vane and his colleagues at the Royal College of Surgeons, London, proposed inhibition of prostaglandin biosynthesis as a mechanism of action for the aspirin-like drugs. In the first paper Vane (1971) showed that aspirin, indomethacin and sodium salicylate inhibited the synthesis of PGE_2 and $PGF_{2\alpha}$ from arachidonic acid in a guinea pig lung cell-free homogenate, in a dose-dependent manner. Secondly, Smith and Willis (1971) showed that the same three drugs inhibited prostaglandin release from human platelets induced by thrombin, without affecting the release of 5-hydroxytryptamine, adenine nucleotide or lysosomal enzymes; furthermore platelets obtained from volunteers who had taken either aspirin or indomethacin were also shown to be capable of very little prostaglandin synthesis when challenged with thrombin. In a third paper, Ferreira, Moncada and Vane (1971) added further weight to the hypothesis by showing that aspirin and indomethacin, though not sodium salicylate, reduced the output of prostaglandins in the perfusate of a dog spleen stimulated with adrenalin.

These pioneering papers triggered off a great surge in prostaglandin research, for here was a group of cheap, readily available, well-studied drugs which were capable of specifically inhibiting PG synthesis in cell-free homogenates, isolated cells and intact organs. Furthermore, the fact that this group of drugs is used extensively in the treatment of chronic human inflammatory disease makes the topic an extremely relevant one to both

pharmacologists and clinicians.

(i) Inhibition by aspirin-like drugs

Within a short space of time Vane's hypothesis had achieved wide acclaim and had been investigated and confirmed in a wide variety of species and preparations (see Flower, 1974 for a comprehensive list). Almost all of the aspirin-like drugs tested have been shown to be potent inhibitors of prostaglandin synthetase, regardless of the source of the enzyme or the analytical technique employed. That inhibition of prostaglandin synthesis is a property solely of the aspirin-like drugs is shown by the fact that many other pharmacologically active compounds are inactive against the enzyme in concentrations of 1.0-5.0mM (Gryglewski and others, 1972).

Salicylic acid is of interest, as it possesses little anti-synthetase activity in vitro, yet is as potent as aspirin as an anti-inflammatory drug, both in animal models and when used therapeutically in man. A possible explanation for this anomaly arises from the observation (Flower and Vane, 1974) that metabolites of salicylic acid are more potent inhibitors of the synthetase system than salicylic acid itself. This point is discussed in detail in section 4.6. Two salicylic acid isomers, m- and p-hydroxybenzoic acid, which possess no anti-inflammatory properties, are both devoid of anti-synthetase activity.

Paracetamol (4-acetamidophenol) was found to be inactive against prostaglandin synthetase prepared from peripheral tissues, but showed good activity against the enzyme prepared from dog, rabbit, mouse or gerbil brain (Flower and Vane, 1972). This is a highly significant finding and raises the possibility that prostaglandin synthetase may exist as iso-enzymes within an organism,

the enzyme from each tissue having its own pharmacolological 'profile' of sensitivity to various aspirin-like drugs. It would thus explain paracetamol's anti-pyretic activity and lack of anti-inflammatory activity. As discussed in section 1.12. the undesirable side-effects of the aspirin-like drugs may be due, at least in part, to the inhibition of gastric and renal prostaglandin synthesis. The design of a drug directed specifically at the synthetase system of the inflammatory 'target' tissue, yet possessing little activity against the enzyme from other tissues may therefore go some way towards solving the major toxicity problems of this group of drugs. Marked discrepancies exist in the literature however on this point. Thus whilst Bhattacherjee and Eakins (1974) found orders of magnitude differences in the potency of indomethacin against the synthetase from various rabbit tissues, Pong and Levine (1976) found virtually identical potencies for seven different rabbit tissues studied, and suggested that previously reported differential sensitivities to indomethacin probably reflected different substrate concentrations in the relatively crude enzyme preparations used. Clearly clarification of this point is highly desirable if the search for an 'ideal' aspirin-like drug is not to be an illusory one.

As the aspirin-like drugs are thought to be competitive inhibitors of prostaglandin synthetase (Ku and Wasvary, 1973), it is perhaps not surprising that the absolute potencies of the drugs reported by different workers vary widely, as a wide range of substrate concentrations have been employed. Even relative potencies reported for the same tissue show great variation (Ferreira and Vane, 1974), so that comparisons between different laboratories can only be made with great caution.

Another factor which influences the activity of the aspirin-like drugs is the way in which the enzyme is prepared. Thus, whilst fluorindomethacin and indomethacin competitively inhibit fresh microsomal preparations from ovine and bovine seminal vesicular glands (Hamberg, 1972), the effect on an acetone powder preparation from sheep vesicular glands was irreversible and increased by pre-incubation (Smith and Lands, 1971).

Evidence in support of Vane's hypothesis was provided by the finding that the rank order of potency of the aspirin-like drugs in the carrageenin rat paw oedema test was the same as the order of potencies as inhibitors of dog spleen synthetase (Flower and others, 1972). An even more striking correlation is shown by studying optical isomers of naproxen, indomethacin and ibuprofen; in each case the isomer with the anti-inflammatory activity shows good anti-synthetase activity and the one with weak anti-inflammatory potency shows little anti-synthetase activity (Ham and others, 1972; Tomlinson and others, 1972; Adams, Bresloff and Mason, 1975). This is a remarkable correlation between an in vitro property and known in vivo potency which no other hypothesis has been able to demonstrate. Clearly one should not expect perfect correlation between in vitro and in vivo properties of a series of drugs, as an orally administered drug faces many hazards of absorption, protein binding, distribution metabolism and excretion before reaching its target in the form of a microsomal enzyme.

In addition to inhibiting PG synthesis in isolated cells and tissues the aspirin-like drugs are capable of reducing whole body PG synthesis. Hamberg (1972), measuring a principal urinary metabolite of PGE_1 and PGE_2 as an index of whole body PG synthesis,

showed that the daily excretion of this compound was greatly reduced in volunteers taking therapeutic doses of aspirin, indomethacin or salicylate. The same experiment was carried out in guinea pigs though in this species the dose required to abolish output of the urinary metabolite was much higher than in man.

(ii) Inhibition by other anti-rheumatic drugs

Greaves and McDonald-Gibson (1972) reported inhibition of PG synthesis in rat skin homogenates by some steroidal anti-inflammatory drugs, though later work using a microsomal preparation showed this group of compounds to be inactive, and it is now generally agreed that steroidal anti-inflammatory drugs do not inhibit prostaglandin synthetase. Recent work however suggests that these drugs may block an earlier stage of prostaglandin production, namely the release of substrate (arachidonate) from cellular phospholipids (Hong and Levine, 1976; Nijkamp and others 1976). Studies of the effects of steroidal anti-inflammatory drugs on PG production by rheumatoid synovial cultures and cultured mouse fibrosarcoma cells also support this conclusion. In the former study (Kantrowitz, Robinson and McGuire, 1975) it was shown that cortisol, dexamethazone and prednisolone, when present in culture fluid at concentrations of 10^{-8}M to 10^{-4}M inhibited the production of prostaglandins in a dose-related manner. This inhibition of PG production was not due to inhibition of PG release, as has been suggested by others (Lewis and Piper, 1975), nor was it due to enhanced PG metabolism in the tissue or degradation in the culture medium. In the latter report (Tashjian, Voelkel and McDonough, 1975) cortisol was shown to be a potent ($ID_{50} \sim 10^{-8}$M) inhibitor of PG formation by a clonal strain of cells in culture, and the effect was not the result of an action on PG release or transport mechanisms.

Chloroquine has been reported to inhibit prostaglandin synthetase, though only at high doses (Gryglewski and others, 1972). Nugteren and others (1966) and Deby, Bacq and Simon (1973) have proposed that the therapeutic activity of some gold salts could be due to their action on the prostaglandin synthetase system. Penicillamine has proved to be inactive as a PG synthetase inhibitor.

(iii) Inhibition by substrate analogues and fatty acids

Ahern and Downing (1970) reported a time-dependent inhibition of sheep vesicular gland synthetase by an acetylenic analogue of arachidonic acid, eicosa-5, 8, 11, 14-tetraynoic acid, TYA. The inhibition was irreversible and the acid was not a substrate for the enzyme. Nugteren (1970) showed that a 12-trans analogue of dihomo-γ-linolenic acid and a 12-trans analogue of arachidonic acid were unusually potent inhibitors of PG synthesis; both were competitive inhibitors and were unchanged by the enzyme. Robak, Dembinska-Kiec and Gryglewski (1975) showed that a series of saturated fatty acids, ranging from C_{10} to C_{18} were capable of inhibiting the microsomal synthetase system of bovine seminal vesicles in vitro, the most potent being lauric and myristic acid. Capric acid had previously been reported to be a weak inhibitor (Wallach and Daniels, 1971).

All these compounds, though useful in the elucidation of enzyme mechanisms, are of little therapeutic interest and have rarely been used in vivo, owing to incomplete knowledge of their absorption, excretion, distribution and metabolism. Certain prostaglandin analogues have been shown to competitively inhibit PG synthesis (McDonald-Gibson, Flack and Ramwell, 1973), suggesting that a product binding site, as well as a substrate site, exists on the enzyme, as postulated by others (Lands and others, 1973).

(iv) Inhibition by miscellaneous agents

In common with many other enzymes, PG synthetase may be inhibited by excessive amounts of cofactors or substrate. Nugteren and others (1966) showed that PGE_1 production could be inhibited by excess amounts of reduced glutathione and other anti-oxidants and Flower, Cheung and Cushman (1973) demonstrated substrate inhibition for PGE_2 production above a concentration of 0.5mM. Some metal ions, including Zn^{2+}, Cd^{2+} and Cu^{2+} have been shown to be inhibitory (Nugteren and others, 1966) though many others were without effect at the concentration used (5×10^{-5}M). Lee and Lands (1972) showed that the inhibition of PGE synthesis by Cu^{++} was accompanied by a simultaneous increase in PGF synthesis. This finding has possible therapeutic implications and will be discussed in detail in section 4.4. Many other entities are able to influence PG synthesis and have been comprehensively reviewed by Flower (1974).

1.12 Side effects of aspirin-like drugs

The aspirin-like drugs share the common unwanted side effects of causing gastrointestinal irritation which may lead to ulceration, and varying degrees of nephrotoxicity with some incidence of papillary necrosis. As this group of drugs is used for the alleviation of chronic inflammatory conditions these side effects often impose a serious limitation on their therapeutic usefulness. It is possible that these toxic effects may be caused by the inhibition of PG synthesis in tissues which depend upon prostaglandins for the control and maintenance of normal physiological function.

PGE_1 inhibits gastric acid secretion (Shaw and Ramwell, 1968)

and may therefore play a part in the control of acidity in the stomach; inhibition of this locally released PG by the ingestion of aspirin-like drugs could therefore lead to hyperacidity and possible ulceration. Several of the synthetic PG analogues have been shown to be potent inhibitors of gastric acid secretion and may have clinical potential as anti-ulcer compounds (Gallus and Hirsh, 1976). Locally released prostaglandins in the stomach may also exert a protective effect on the mucosa by increasing its blood supply, the removal of this function by aspirin-like drugs leading to ischaemia and necrosis. A further possibility is that ingestion of aspirin-like drugs can lead to a local accumulation of arachidonic acid and this, or possibly a peroxide derivative, could cause gastric irritation; in this context prostaglandins may be viewed as mere metabolic waste, consequent upon the removal of potentially damaging lipids as suggested provocatively by Brocklehurst and Dawson (1974).

In the kidney prostaglandins are natriuretic (Lee, 1972), prostaglandin synthetase being located in the cells forming the collecting tubules (Janszen and Nugteren, 1971). Prostaglandin synthesis and release in the kidney may be important for the local control of both blood and urine flow; infusion of angiotensin into the kidney releases a prostaglandin which moderates the vasoconstriction and indomethacin, by inhibiting PG synthesis, effectively potentiates the vasoconstrictor effects of angiotensin, producing a much larger decrease in renal blood flow (Vane, 1972).

1.13 Objects of the study

The work described in this thesis revolves around the possible involvement of prostaglandins in the pathogenesis of

rheumatoid arthritis, and the hypothesis that aspirin-like drugs exert their therapeutic effects via inhibition of prostaglandin biosynthesis.

On the basis of studies of the interaction between aspirin-like drugs and prostaglandin synthetase prepared from various animal tissues, many authors have gone on to discuss the anti-inflammatory actions of these drugs in the treatment of rheumatoid disease. This is a little surprising in view of the fact that the same authors apparently accept the hypothesis that isoenzymes of prostaglandin synthetase exist, and that even within the same species the enzymes from different tissues may have widely differing susceptibilities to inhibition by aspirin-like drugs. When the present study was initiated there was no evidence in the literature to suggest that results obtained using animal preparations of prostaglandin synthetase could be extrapolated to human inflammatory tissue. The logical conclusion to be drawn, therefore, was that if meaningful results were to be obtained on the interaction between aspirin-like drugs and prostaglandin biosynthesis in rheumatoid disease, the enzyme used in the studies should be derived from tissue involved in the inflammatory reaction.

In a preliminary study in this laboratory, Rose (1975) was unable to demonstrate any prostaglandin synthetase activity in a cell-free homogenate of rheumatoid synovium, though initial results obtained by the present author suggested that this was an unsuitable preparation to use in combination with the assay system employed. The work described in this thesis was therefore carried out in order to answer or clarify as many as possible of the following points :-

1. Is rheumatoid synovium capable of prostaglandin biosynthesis?

2. Do its fundamental biochemical properties show it to be radically different from the well-studied enzymes prepared from animal tissues?

3. Does the level of enzyme activity found in rheumatoid synovium correlate with other parameters of disease activity, either clinical or biochemical?

4. How does the enzyme activity in rheumatoid synovium compare with levels found in other tissues?

5. Does R.A. synovium contain raised levels of enzyme activity compared with normal synovial tissue?

6. Is the enzyme susceptible to inhibition *in vitro* by the aspirin-like drugs used therapeutically?

7. If so, is this inhibition achieved at therapeutically 'realistic' concentrations?

8. Does the potency of the aspirin-like drugs as inhibitors of rheumatoid synovial prostaglandin synthetase correlate with their known therapeutic potencies, i.e. would this assay form the basis of a good *in vitro* screening test for new anti-inflammatory drugs?

9. Do other antirheumatic drugs, e.g. steroids, penicillamine, have any effect on prostaglandin biosynthesis in this system?

10. Which cells are capable of contributing to PG production at the inflammatory site?

11. Do all the aspirin-like drugs inhibit the enzyme in the same manner?

12. At which point in the synthesis of prostaglandins from arachidonic acid do the aspirin-like drugs act?

13. Do any of the aspirin-like drugs show 'selective' inhibition of any one product of arachidonic acid metabolism?

14. Can any information concerning the structure or organisation of the enzyme complex be gained from studying the interaction between the enzyme and aspirin-like drugs?

15. In summary, do the results of the study as a whole support or detract from the hypothesis that aspirin-like drugs exert their pharmacological effects via inhibition of PG biosynthesis?

CHAPTER TWO

METHODOLOGY

2.1 Introduction

A multitude of techniques are now available for the separation, purification, identification and quantitation of prostaglandins.

Chromatographic techniques have been described for preliminary purification and separation of prostaglandins including thin layer chromatography (Green and Samuelsson, 1964), paper chromatography (Egan, 1975), silica gel microcolumns (Hillier and Dilley, 1974), sephadex LH-20 columns (Zia and Horton, 1973), ion-exchange liquid chromatography (Morozowich, 1974) and high pressure liquid chromatography (Andersen and Leovey, 1974).

Identification and quantitation may be achieved using bioassay (Ferreira and Vane, 1967), radioimmunoassay (Levine, 1973), competitive protein binding (Frölich and others, 1974), viroimmunoassay (Andrieu, Mamas and Dray, 1974), radioisotope dilution (Aizawa and Yamada, 1974), enzymatic assay (Matschinsky, Shanahan and Ellerman, 1974), spectrophotometry (Takeguchi and Sih, 1972), isotope derivative assay (Flower and McClure, 1975), gas-liquid chromatography (Pugsley and Beilin, 1974) or combined gas-liquid chromatography/mass-spectrometry (Frölich and others, 1975). As these are only representative publications for each technique it is clear that many combinations of these procedures are possible, and that no particular one is a 'method of choice'.

The initial work involved in adopting and validating a technique can represent a considerable outlay in terms of time and effort before any experiments can be undertaken and results obtained with any confidence. At the start of this work, therefore, careful consideration was given to the many techniques available in order to decide which would be most suitable for the planned

experiments in terms of accuracy, specificity, sensitivity, convenience and cost. It is always recognised that such a choice must inevitably represent a compromise - the most accurate, sensitive and specific technique available is rarely the most convenient.

Bioassay of prostaglandins, using cascade superfusion systems, has been widely used as it is capable of good sensitivity without the need for time-consuming purification of the sample or expensive equipment. Specificity may be increased by the use of antagonists and the use of several tissues in parallel (usually rat stomach strip, rat colon and chick rectum). The disadvantages of the technique are that metabolites cannot usually be measured and the precision of the assay is variable.

Radioimmunoassay has been increasingly used by many workers due to its excellent sensitivity, potential ability to measure any prostaglandin or metabolite, and the feasibility of assaying large numbers of samples. Elegant studies by Levine (1973) have shown that the combined use of several antibodies of different specifities may yield much information concerning the kinetics of prostaglandin metabolism. The fundamental objection to radioimmunoassay has always been its inherent lack of specificity - however exhaustive the cross-reactivity studies performed to validate the specificity, the possibility always exists that a new, as yet unknown prostaglandin or prostaglandin metabolite (or even a non-prostanoid compound) may bind to the antibody. That this is not merely a hypothetical objection has been shown in work by Samuelsson (1973) and Frölich and others (1975). In the former study the assay of $PGF_{2\alpha}$ levels in peripheral venous blood, using both a radioimmunoassay and a GC/MS method, has shown that much of the material

binding to $PGF_2\alpha$ antibodies is not in fact $PGF_2\alpha$.
In the latter work, also employing a GC/MS method, much doubt has been cast on previously obtained values for PGA_2 levels in human plasma - the values obtained by GC/MS were only about 10% of those previously reported using a radioimmuncassay technique. A further drawback of adopting a radioimmunoassay technique is the time (and a certain element of luck) involved in producing the appropriate antibodies - often several months before a good titre of high specificity antibody is obtained.

Gas-chromatography / mass spectrometry is seen by many as the method of choice for the identification and quantitation of prostaglandins in tissue extracts and biological fluids. It is an accurate, sensitive technique and the presence of interfering compounds is easily detected. The technique is also applicable to metabolites, and the use of deuterated internal standards and single-ion monitoring allows accurate quantitation in the low nanogram range. This technique would thus be widely used, were it not for the high cost of the equipment and the extremely time-consuming preparatative and technical procedures involved in its operation.

Spectrophotometric techniques for the measurement of prostaglandins have the attraction of simplicity. Their sensitivity, however, is not good and at low levels specificity may be doubtful. Furthermore, this method can only be used for prostaglandins which are natural chromophores e.g. PGA, PGB or those which can be easily converted to a chromophore e.g. PGE. Spectrophotometric methods have been successfully used to measure relatively high concentrations of incubation products where high substrate concentrations were employed with very active prostaglandin synthetase preparations

from ovine or bovine seminal vesicular glands (Takeguchi and Sih, 1972). However, as discussed later, it was considered of prime importance in this work to use 'realistic' substrate concentrations approximating to those which exist in vivo i.e. very low concentrations of substrate (~1μM) producing only nanogram amounts of products.

As the purpose of this work was primarily to study prostaglandin synthetase activity in rheumatoid tissue, rather than to measure concentrations of endogenous prostaglandins in synovial fluid or synovial tissue, consideration was given to the possibility of employing a radiometric assay to measure the enzyme activity. Previous experience with a radiometric assay for serum lecithin-cholesterol acyl transferase (LCAT) activity (Misra and others, 1974) had convinced the author that such a technique was reliable and convenient, possessing good accuracy, precision and sensitivity, even using ^{14}C labelled substrate of relatively low specific activity.

The proposed method for use in this study would thus involve incubation of prostaglandin synthetase, from synovial tissue, with radioactively labelled substrate (either ^{14}C or ^{3}H), followed by extraction and separation of unchanged substrate and formed prostaglandins (and possibly other, non-prostanoid, products). Accurate and sensitive measurement of the labelled prostaglandins formed, and hence quantitation of the enzyme activity, would be achieved by liquid scintillation counting.

At the time this work was undertaken, ^{3}H-labelled substrate (arachidonic acid) of high specific activity was not commercially available. However, calculation showed that the ^{14}C arachidonic acid available (Radiochemical Centre, Amersham) was of sufficiently high

specific activity to achieve good sensitivity, and would have the further advantage of greater radiochemical stability (^{3}H labelled compounds can be troublesome due to tritium exchange in solution). Of the commonly used methods available for separation of unchanged substrate and formed prostaglandins, thin-layer chromatography was chosen rather than paper or column chromatography due to its speed, ease of handling and ease of radioscanning to locate active zones prior to liquid scintillation counting.

2.2 Materials

The reagents used in these studies were purchased from British Drug Houses Ltd., Poole or the Sigma Chemical Company Ltd., London. Whenever possible they were of 'Analar' grade or its equivalent. Solvents to be used for storage purposes (benzene or ethanol) were redistilled through a 60cm packed fractionation column. Pyridine used in the synthesis of {^{3}H-acetyl} aspirin was redistilled immediately before use.

Silica gel G was purchased from Merck, Darmstadt, Germany and scintillation chemicals from Koch-Light Laboratories, Colnbrook. Disposable polythene liquid scintillation counting vials were supplied by Hos-Tek Ltd., Canterbury.

The following radiochemicals were obtained from the Radiochemical Centre, Amersham :-

^{3}H-Acetic anhydride	Specific activity	3,500mCi/mmol
{1-^{14}C} Arachidonic acid	" "	56mCi/mmol
{5,6,8,11,12,14,15(n)-^{3}H} Prostaglandin E_2	" "	140,000mCi/mmol
{5,6,8,11,12,14,15(n)-^{3}H} Prostaglandin $F_{2\alpha}$	" "	150,000mCi/mmol

The prostaglandin standards used in this work were the generous gift of Dr. J.E. Pike, Upjohn Co., Kalamazoo, U.S.A.

The following drugs used were the generous gifts of the manufacturing companies :-

Chemical name	Trade name	Company
Indomethacin	Indocid	Merck, Sharp & Dohme
Ibuprofen	Brufen	Boots Pure Drug Co.
Flurbiprofen	Froben	
Naproxen	Naprosyn	Syntex
Azopropazone	Rheumox	A. H. Robins
Flufenamic acid	Arlef	Parke, Davis & Co.
Mefenamic acid	Ponstan	
Phenyl butazone	Butazolidin	Geigy Pharmaceuticals
Ketoprofen	Orudis	May & Baker
Benorylate	Benoral	Winthrop Laboratories
Fenclofenac	-	Reckitt & Colman

1-^{14}C-Arachidonic acid was diluted to a concentration of 1μCi / ml with re-distilled benzene and stored under nitrogen at 4^{o}C in dark, sealed ampoules. Stock solutions of non-radioactive arachidonic acid were prepared in redistilled benzene at concentrations of 10mg/ml and 0.1 mg/ml and stored under nitrogen at -20^{o}C in dark, sealed ampoules. When used for enzyme studies, the benzene was evaporated under nitrogen at 37^{o}C, and the residue dissolved in a small volume of redistilled absolute ethanol (20-50μl). This ethanolic arachidonic acid solution was diluted to the desired concentration with tris-acetate buffer, pH8.0 to give an ethanol concentration which did not exceed 1%.

All glassware which had been in contact with radioactive extracts was soaked in chromic acid overnight, washed thoroughly and rinsed with distilled water and acetone.

2.3 Preparation of microsomal PG synthetase

(i) Bovine seminal vesicles

A freeze-dried microsomal powder of bovine seminal vesicles was prepared according to the method of Takeguchi, Kohno and Sih (1971) and was the generous gift of Dr. P. Bresloff, Boots Pure Drug Co., Nottingham.

(ii) Rabbit renal medulla

Albino Californian rabbits of both sexes, 2-3kg. body weight, were killed by cervical dislocation. The kidneys were immediately excised, and after removal of the adhering fat and connective tissue were placed on ice. Each kidney was cut into lateral slices 2-4mm thick with a scalpel blade, and the medullary tissue dissected from the cortex with scissors. The medullae from both kidneys, 2-6 gm, were coarsely chopped before homogenisation (20% w/v) in 0.1M tris-acetate buffer, pH 8.0 containing sucrose (0.25M), EDTA (1mM), hydroquinone (0.5mM), cysteine hydrochloride (1mM) and reduced glutathione(2mM)., using a Potter-Elvehjm homogeniser. The homogenate was centrifuged for 10 min. at 4000g, filtered through cheesecloth and centrifuged for a further 10 min. Centrifugation of the supernatant at 10^5g for 1h. produced a microsomal pellet which was used as the enzyme source.

(iii) Human rheumatoid synovium

Synovial tissue, obtained at surgical synovectomy of the knee or elbow, or at total knee replacement, was kept on ice and used within one hour. (No attempt was made during this work to

utilise tissue which had been either deep frozen or stored at 4^{o}c. due to the intrinsic instability of the enzyme). The synovium was dissected free of fat and chopped into small pieces with scissors. In preliminary studies, the tissue was finely minced and a 20% (w/v) homogenate prepared in 0.1M tris-acetate buffer, pH 8.0, containing sucrose (0.25M), EDTA (1mM), hydroquinone (0.5mM), cysteine hydrochloride (1mM), and reduced glutathione (2mM), using a combination of a Waring blender and a Potter-Elvehjm homogeniser. Subsequently a shaft-type homogeniser was acquired (1L Model X-1020, Scientific Instrument Centre Ltd., London) and this was found to give a more efficient homogenisation and hence greater yield of microsomes. Using this instrument, tissue was homogenised at full speed for 1 minute, while cooled in ice. The homogenate was centrifuged for 10 min. at 4000g and the supernatant filtered through a double layer of cheesecloth. This process was repeated, and a microsomal pellet obtained by centrifugation at 10^{5}g for one hour. The average yield of microsomes was 1.0mg protein/g.tissue.

(iv) Human peripheral leucocytes

(a) 'Total' white cell preparation

Venous blood was taken into heparin (20 units/ml) and centrifuged at 600g for 10 min., at room temperature. The plasma and buffy white cell layer were then pipetted into a 20ml plastic syringe. After incubation at 37^{o}c for 1 hour, the turbid plasma layer was transferred to plastic tubes and centrifuged at 600g for 10 min. The pellet was washed twice in phosphate-buffered saline (P.B.S.), then resuspended in P.B.S. (5.0ml) and a cell count performed. After obtaining a cell pellet again by centrifugation, ice-cold tris-acetate buffer (as used in (iii) above) was added (5.0ml) and the suspension sonicated (Rapidis

300, Ultrasonics Ltd) by three 30 second impulse of 200 watts. Cell debris and any intact cells were removed by centrifugation (2000g for 10 min), after which a microsomal pellet was obtained by centrifugation at 10^5g for one hour.

(b) Platelets

Platelet-rich plasma was obtained by centrifugation of heparinised blood at 50g for 10 min., then at 100g for 10 min. A platelet count was performed and a pellet obtained by centrifugation of the plasma at 2000g for 5 min. The platelets were sonicated and a microsomal pellet obtained as described above for the 'total' white cell preparation.

(c) Polymorphonuclear cells

The method used was based on that of Dioguardi and others, (1963). Heparinised blood (15 ml) was added to 0.83% NH_4Cl (70ml), gently mixed and allowed to stand at room temperature for 10 min. After centrifugation at 120g. for 10 min. the supernatant was discarded and the washing process with NH_4Cl repeated. After this stage the preparation was free of erythrocytes. Residual lymphocytes were removed by suspending the pellet in Hank's solution (50ml) and centrifuging at 55g for 10 min. Lymphocytes, suspended in the supernatant, were discarded and the washing repeated twice. Typically, the final PMN preparation contained 92% PMN, 6% lymphocytes and 2% monocytes. The yield was approximately 65-70% of the original cell number and PMN viability > 98% as judged by trypan blue exclusion. (Phillips, 1973). The PMN cells were sonicated and a microsomal pellet obtained as described above for the 'total' white cell preparation.

(d) Lymphocytes

The method used was essentially as described by Boyum (1968). Heparinised blood (10ml) was added to a solution of 1% methyl cellulose in 0.9% saline (2.5ml), 30mg carbonyl iron added and the mixture gently shaken at 37°c for 30 min. The blood bottle was then stood on a strong magnet for 30 min. in a 37°c incubator, during which time the erythrocytes, together with the iron-loaded PMN's and large mononuclear cells, sedimented out.

Triosil (Nyegaard & Co., Oslo; 20 ml) was diluted with water (24ml), mixed well and added to 9% ficoll (100ml). The lymphocyte-rich supernatant was carefully removed after the 37°c incubation, layered onto the ficoll-triosil solution and centrifuged at 220g for 30 min. The lymphocytes, appearing as a white band at the interface between the upper plasma layer and the lower ficoll-triosil, were carefully removed and washed twice in Hank's physiological saline. Contamination of this preparation by PMN's rarely exceeded 2% and cell viability, as above, was >98%.

(v) Synovial fluid leucocytes

Fresh samples of synovial fluid, obtained from R.A. patients, were incubated with hyaluronidase (1.5% $^{v}/_{v}$ of a 0.02% solution) at 37° for 30 minutes. A white cell pellet was obtained by centrifugation at 4000g for 10 minutes, after which a microsomal fraction was prepared as described above in (iv), (a) for peripheral leucocytes.

2.4 Production of prostaglandins from ^{14}C arachidonic acid substrate

Microsomal PG synthetase, prepared as described in section

2.3 above was resuspended in fresh tris-acetate buffer, pH 8.0 (see section 2.3(iii) above), by a brief period of sonication (The use of a 'Whirlimixer' did not give an efficient re-suspension of the microsomal pellet). Aliquots of the microsomal suspension were incubated with ^{14}C arachidonic acid (100nCi/0.1ml; final concentration 0.85μM) at 37°c, for one hour, with shaking, in a volume of 2.0ml. The reaction was terminated by the addition of 2M citric acid solution (0.25ml), reducing the pH to 2.5-3.0.

2.5 Extraction, separation and quantitation of ^{14}C prostaglandins

The formed ^{14}C prostaglandins and unconverted ^{14}C arachidonic acid were extracted from the acidified incubate with diethyl ether (2x3.0ml). After evaporation of the combined extracts under nitrogen at 37°c the residue was immediately dissolved in 20μl chloroform:methanol, 2:1, and 5μl applied to a thin-layer chromatographic plate (silica gel G, 0.25mm thickness, containing 2.5% silver nitrate). Authentic arachidonic acid, PGE_2 and $PGF_{2\alpha}$ (10μg of each) were applied in a marker lane and the plate developed in the solvent system benzene-dioxan-acetic acid, 20:20:1 (system AI) to a height of 15 cm. After locating the markers with phosphomolybdic acid, and scanning the plate for radioactivity (Berthold thin-layer scanner, Model LB 2723, Camlab, Cambridge), the zones in the extract lanes which corresponded to the markers were scraped from the plate, transferred to counting vials and methanol (1.0ml) added. After shaking briefly, 8ml of liquid scintillant was added (0.5% PPO and 0.03% dimethyl POPOP in toluene) and the samples counted in a liquid scintillation system (Phillips Liquid Scintillation Analyser, Model 01). Samples were counted at their 'balance point' to a present level of 10^4 c.p.m. (error ± 2%), at an efficiency of approx. 85%. The absolute activity (d.p.m.) was

calculated by the counter, using a preprogrammed 'quench correction curve' based on a channels ratio (see appendix 1). From the counts obtained in the substrate and prostaglandin regions of the chromatogram (which represent approx. 95% of the total activity on a typical separation), the percentage conversion of the substrate can be calculated, and hence the activity of the microsonal PG synthetase preparation derived :-

$$\text{Activity} = \frac{\%\ \text{substrate conversion} \times \text{substrate concentration}}{100 \times \text{microsomal protein concentration}}$$

Throughout this study, the PG synthetase activity has been expressed in terms of 'pmol PG produced per mg. microsomal protein per hour'.

2.6 Characterisation of formed prostaglandins

The formed prostaglandins were characterised by running in various chromatographic systems, with authentic prostaglandin standards, by chemical conversion to other prostaglandins followed by further chromatographic identification, and by bioassay of TLC eluates.

(i) Chromatography

The chromatographic systems used were :-

1. Benzene-dioxan-acetic acid, (20:20:1), with and without silver nitrate.
2. Ethyl acetate-methanol-acetic acid-water-2,2,4 trimethyl pentane, (110:35:30:100:10), upper phase used.
3. Chloroform-methanol-acetic acid-water, (90:6:1:0.75).

System 1 was used routinely in this project, systems 2 and 3 being used for confirmation whenever a new tissue or cell preparation

was being characterised. An aliquot of the incubation extract was always added to the authentic marker spot before chromatography, as it was observed that pure markers running in a lane which contained no extract did not run exactly parallel with markers superimposed on an extract lane. This was confirmed by the addition of authentic radioactive prostaglandins to extract and marker lanes, followed by chromatography and radioscanning.

(ii) Chemical conversion of ^{14}C-PGE_2

The radioactive zone in the extract lane which corresponded to authentic PGE_2 was scraped from the thin-layer plate into a small glass sinter column and eluted with redistilled methanol (2.0ml), solid NaCl (10mg) was added, and the precipitated AgCl removed by centrifugation at 2000g for 5 min. The clear supernatant was removed, divided into two portions and evaporated under nitrogen at 37°c. To one portion was added 0.5M NaOH (2.0ml), the tube mixed well and incubated at 37°c for 1h. To the second was added water (2.0ml), the pH adjusted to 8.0 with 0.05M NaOH, solid $NaBH_4$ added (10mg) and the tube also incubated at 37° for 1h. Both mixtures were then brought to pH 2.5 with 2M citric acid, extracted with diethyl ether (3 x 2.0ml) and the residues, after evaporation of the extracts, re-chromatographed in the solvent system benzene-dioxan-acetic acid, (20:20:1).

(iii) Bioassay of TLC eluates

A portion of the chloroform/methanol extract was applied to a thin layer plate (silica gel G, 0.25mm thickness) and developed to a height of 15 cm in the AI solvent system. 1 cm bands were transferred to small glass sinter columns and eluted with redistilled methanol (1.2 ml), an aliquot of which (0.2 ml) was used to measure

the radioactivity present in each extract. The remainder was evaporated under nitrogen at 40°, redissolved in Tyrode's solution (1.0ml) and aliquots (0.1ml) used for bioassay.

A rat stomach strip was superfused with Tyrode's solution containing a mixture of antagonists (Gilmore, Vane and Wyllie, 1968) at a flow rate of 4.5ml/min. Standard solutions of PGE_2 and $PGF_{2\alpha}$ were used for calibration purposes. The use of a rat colon strip in series with the stomach strip, in order to preferentially detect any $PGF_{2\alpha}$ present, was not successful.

(iv) Detection of PG-metabolising activity in microsomal preparations

Although the principal prostaglandin metabolising enzymes are known to be found in the cytoplasmic fraction of cells, the possibility exists that a microsomal preparation might contain, or be contaminated with, a small but significant amount of prostaglandin-metabolising activity. This would clearly be a potential source of error in microsomal incubations, particularly when the effect of PG synthetase inhibitors was being studied. (Indomethacin and aspirin are both inhibitors of 15-hydroxyprostaglandin dehydrogenase (Hansen, 1974).

The possibility that the microsomal preparation might contain PG metabolising activity was therefore investigated by adding authentic ^{3}H-PGE_2 and ^{3}H-$PGF_{2\alpha}$ as substrate, in place of ^{14}C arachidonic acid, to separate microsomal incubates. These were then taken through exactly the same incubation, extraction and separation procedures as for the usual arachidonate incubates. Using the AI solvent system, possible PG metabolites such as the 15-oxo or the 13,14-dihydro-15-oxo metabolites would be readily detectable.

2.7 Protein estimation

Microsomal protein concentrations throughout this thesis were measured by the method of Lowry and others (1951), using bovine serum albumin as standard. Concentrations varied between experiments, but were in the range 0.4-2.0 mg/ml for the synovial studies, and 50-200µg/ml for the white cell studies.

2.8 Preliminary results

Initial studies were carried out in order to check the validity of the method in terms of accuracy and precision and to determine the optimum conditions for extraction, chromatography and liquid scintillation counting.

(i) Extraction

In order to check the completeness of extraction of formed prostaglandins and unchanged substrate from incubates, 100nCi of either ^{14}C-arachidonic acid, ^{3}H-PGE_2 or ^{3}H-$PGF_{2\alpha}$ was added in duplicate to suspensions of R.A. synovial microsomes (2.0ml) exactly as used for incubation studies (section 2.4), the mixture acidified with 2M citric acid (0.25ml) and extracted with three portions of diethyl ether (3.0ml each). Each separate ether extract was evaporated to dryness in a counting vial, methanol (1.0ml) then liquid scintillant (7.0ml) added and the amount of activity present determined by liquid scintillation counting, from which percentage recoveries were calculated (Table 2.1).

Table 2.1 Percentage recoveries of authentic ^{14}C-arachidonic acid, ^{3}H-PGE_2 and ^{3}H-$PGF_{2\alpha}$ from suspensions of R.A. synovial microsomes

	Arachidonic acid		PGE_2		$PGF_{2\alpha}$	
1st ether extraction	94.1 97.9	96.0	91.9 90.7	91.3	85.9 86.8	86.4
2nd ether extraction	3.9 2.3	3.1	6.2 6.3	6.3	10.8 10.5	10.7
3rd ether extraction	0.3 0.1	0.2	0.9 0.9	0.9	1.1 1.1	1.1

As can be seen, all three compounds are readily extracted

from the incubation mixture by diethylether, two extractions being sufficient to extract greater than 97% of each of the three compounds. As would be expected the least polar compound (arachidonic acid) is extracted with slightly greater efficiency than the other two, and vice versa for $PGF_2\alpha$.

The experiment was repeated, except this time the combined ether extracts were evaporated to dryness under nitrogen in small conical tubes, and the residues assayed for radioactively labelled arachidonic acid, PGE_2 and $PGF_2\alpha$ exactly as for a normal incubate (section 2.5). Overall recoveries for the entire procedure for arachidonic acid, PGE_2 and $PGF_2\alpha$ were 92.1%, 90.7% and 88.6% respectively. This was considered to be satisfactory both in terms of absolute recovery of each compound, and of comparative recoveries for the three compounds, permitting calculations of prostaglandin synthetase activity to be made on the basis of the percentage conversion of the substrate to prostaglandins.

(ii) Chromatography

An inspection of the solvent systems available for thin layer chromatography of prostaglandins (Green and Samuelsson, 1964) showed that the AI system (benzene-dioxan-acetic acid, 20:20:1) should give a good separation of arachidonic acid and the anticipated products PGE_2 and $PGF_2\alpha$. Silver nitrate (2.5%) was included in the plates as this was found to give a larger separation between the two prostaglandins than when silica gel alone was used. It was also found that an unlagged, unequilibrated tank gave a much better separation than if the system was equilibrated, as is the usual practice. Typical Rf values obtained using this system were:-

	Rf
$PGF_2\alpha$	0.32
$PGF_1\alpha$	0.38
PGE_2	0.55
PGE_1	0.58
PGD_2	0.60
PGA_2	0.87
PGB_2	0.90
A.A.	0.93

The running time for the system was about 1½ hours, though this was somewhat temperature-dependant and could be as long as 3 hours when the room temperature was low. The solvent itself is stable and can be re-used many times without any adverse effects on either the separation or the chemical stability of the extracts.

(iii) Liquid scintillation counting

Quench corrections were made with the use of a channels-ratio method, owing to the heterogeneous nature of the samples to be counted. A series of standard vials were prepared using hexadecane-1-^{14}C (5,170d.p.m.), silica gel (scraped from an area 2.0cm x 1.5cm of a 0.25mm thick plate), methanol (1.0ml), toluene scintillant (7.0ml) and varying amounts of carbon tetrachloride (0-150µl) as the quenching agent. With one channel of the counter set for balance point counting, a second channel's attentuation was varied and the counts for each sample determined simultaneously in both channels. The ratio of the count in each channel was plotted against the efficiency of counting in the channel set for balance point counting. The attenuation setting was selected which gave the nearest to a straight line calibration, and the parameters of this line set on

the computer calculator boards of the instrument. Typical efficiencies found for incubate extracts were in the range 85-91%.

(iv) Precision of the assay

During these initial experiments all assays were carried out in duplicate in order to obtain a measure of the precision of the method. The index of precision, (s), for duplicate determinations was calculated from the formula :-

$$s = \sqrt{\frac{\Sigma d^2}{2n}}$$

where d is the difference between duplicates and n the number of duplicates.

For prostaglandin synthetase activities in the range 146 - 395 pmol/mg/hr. it was 19 pmol/mg/hr., (n = 17), a surprisingly good value in view of the relative complexity of the method. In many of the later studies (especially the drug inhibition dose-response curves) duplicate assays could not be performed owing to shortage of the microsomal enzyme preparation, though from the figures above a reasonable estimate of the error on a single determination would be 5-10%.

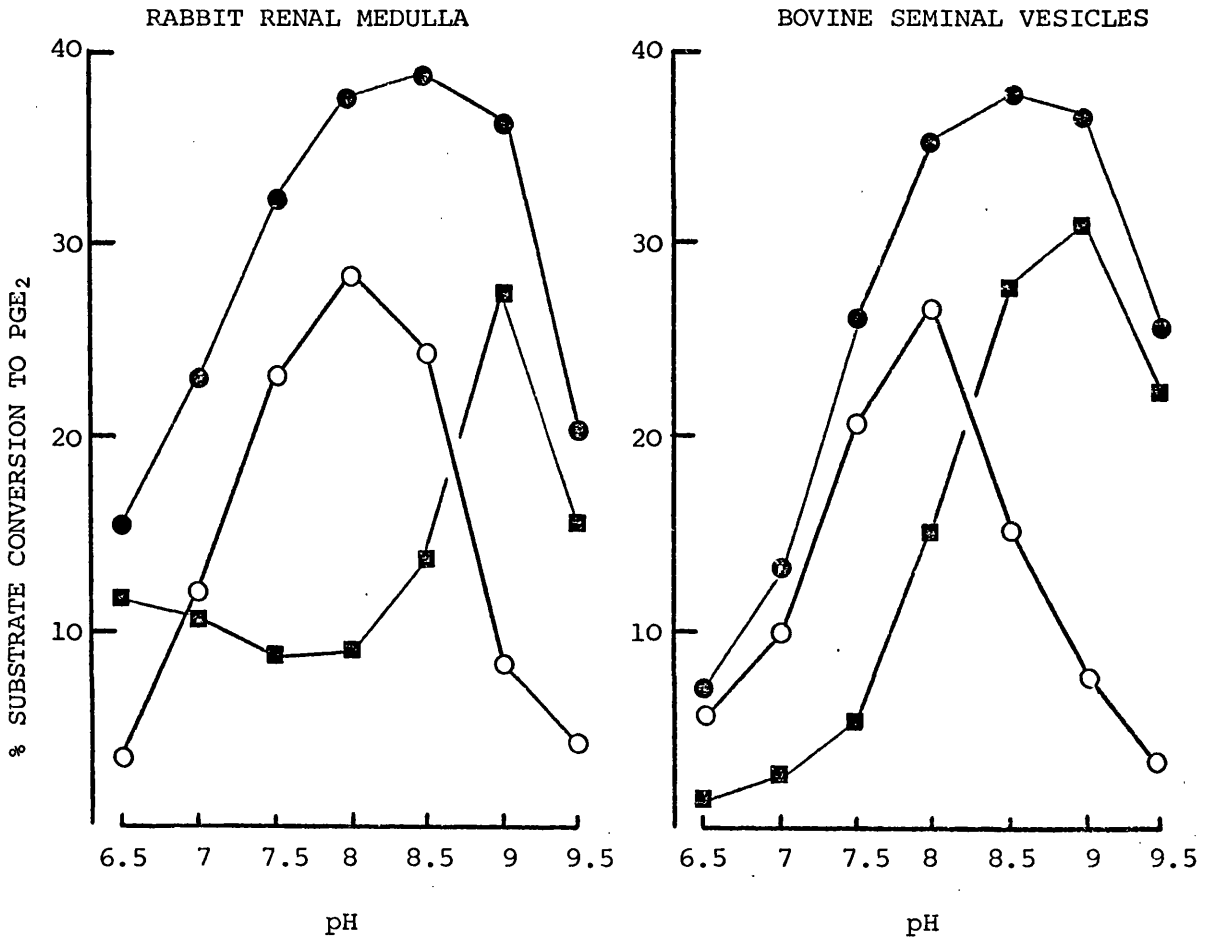
(v) Supernatant 'inhibition'

Previous studies in this laboratory (Rose, 1975) had demonstrated an apparent pH-dependent inhibition of prostaglandin synthesis when the high-speed supernatant fraction of rabbit renal medulla was added back to the microsomal fraction derived from this tissue. In view of the subsequent failure to demonstrate any PG synthetase activity in a cell-free homogenate of human R.A. synovium in the above studies, this phenomenon was briefly re-investigated in order to determine whether a cell-free homogenate of R.A. synovium would be a suitable preparation to use.

To microsomal preparations from bovine seminal vesicles and rabbit renal medullae (section 2.3) was added the high-speed supernatant fraction from rabbit renal medullae (2.8 mg protein/ml) and the mixtures incubated with ^{14}C-arachidonic acid over a pH range. Control incubations were also made over the same pH range, but with the addition of 0.1M tris-acetate buffer, pH 8.0 instead of the rabbit medulla supernatant fraction. The results (Fig 2.1) show that the supernatant fraction produces a significant inhibition of prostaglandin synthetase from both bovine seminal vesicles and rabbit renal medullae, and that the maximum inhibition, in absolute terms, is produced at pH 8.0 in both preparations as shown by the 'difference' curves obtained by subtracting the 'inhibited' value from the supernatant-free value at each pH. It is of interest to note that phospholipase A_2 from rat liver has a pH optimum at pH 8.0 (Bjørnstad, 1966) and pancreatic phospholipase A_2 has a pH optimum in the region 7.9 - 8.4 (de Haas and others, 1968).

A possible explanation of these results is that phospholipase A_2 present in the supernatant fraction releases arachidonic acid (or other polyunsaturated fatty acids) from phospholipids and that this non-radioactive substrate effectively dilutes the radioactive substrate, producing an apparent inhibition of PG synthesis, as only the labelled fraction of the product is measured; this could presumably be verified by measuring the 'total' i.e. labelled plus unlabelled, PG produced over the pH range using bioassay or radioimmunoassay. Although not pursued further these results showed that a cell-free homogenate is not a suitable tissue preparation of the enzyme to use in conjunction with the radiometric assay technique and that the proposed studies of R.A. synovial PG synthetase should be carried out using a microsomal preparation.

Fig. 2.1 The effect of rabbit renal medulla supernatant on PG synthesis by rabbit renal medulla microsomes and bovine seminal vesicle microsomes



Addition of rabbit renal medulla high-speed supernatant (2.8 mg protein/ml) to either rabbit renal medulla microsomes (0.5ml supernatant added per incubation) or bovine seminal vesicles (0.1 ml added) over a pH range produced curves (■——■) having maxima at pH 9.0. Subtraction from control incubations (●——●) containing no supernatant gave curves (O——O) representing the absolute reduction in PG production at each pH value.

CHAPTER THREE

BIOCHEMICAL STUDIES OF RHEUMATOID SYNOVIAL PG SYNTHETASE

3.1 Introduction

Much evidence now exists which suggests that prostaglandins have an important role as mediators or modulators of many aspects of the inflammatory process. Their ability to initiate or augment all the cardinal signs of inflammation together with their seemingly inevitable presence at any site of tissue injury is readily demonstrated in many types of acute inflammation, though their role in the transition from such acute states to the type of chronic, degenerative inflammation typified by rheumatoid arthritis is less obvious. The possible influence of prostaglandins on fundamental aspects of the pathogenesis of chronic inflammatory disease such as granuloma formation, cartilage metabolism and bone resorption is clearly of great interest, though further work will be necesary to relate these initial *in vitro* findings to the situation *in vivo*.

Aspirin-like drugs form the backbone of antirheumatic therapy, and it was therefore of great interest to pharmacologists and clinicians alike when Vane (1971) proposed that this group of drugs exert their pharmacological effects by inhibiting prostaglandin biosynthesis. This hypothesis acted as a great stimulus for further research into the interaction between aspirin-like drugs and prostaglandin biosynthesis, and many reports of both *in vivo* and *in vitro* studies soon substantiated the initial findings. As emphasised by Whitehouse (1965) there are great advantages in employing an *in vitro* technique as a screen for anti-inflammatory activity, and it soon became *de rigeuer* to test potential new anti-inflammatory drugs for their ability to inhibit prostaglandin biosynthesis *in vitro*. Such studies have invariably been carried out using the enzyme derived from ovine or bovine seminial vesicular glands as these (microsomal) preparations are convenient to use in lyophilised form and are highly active.

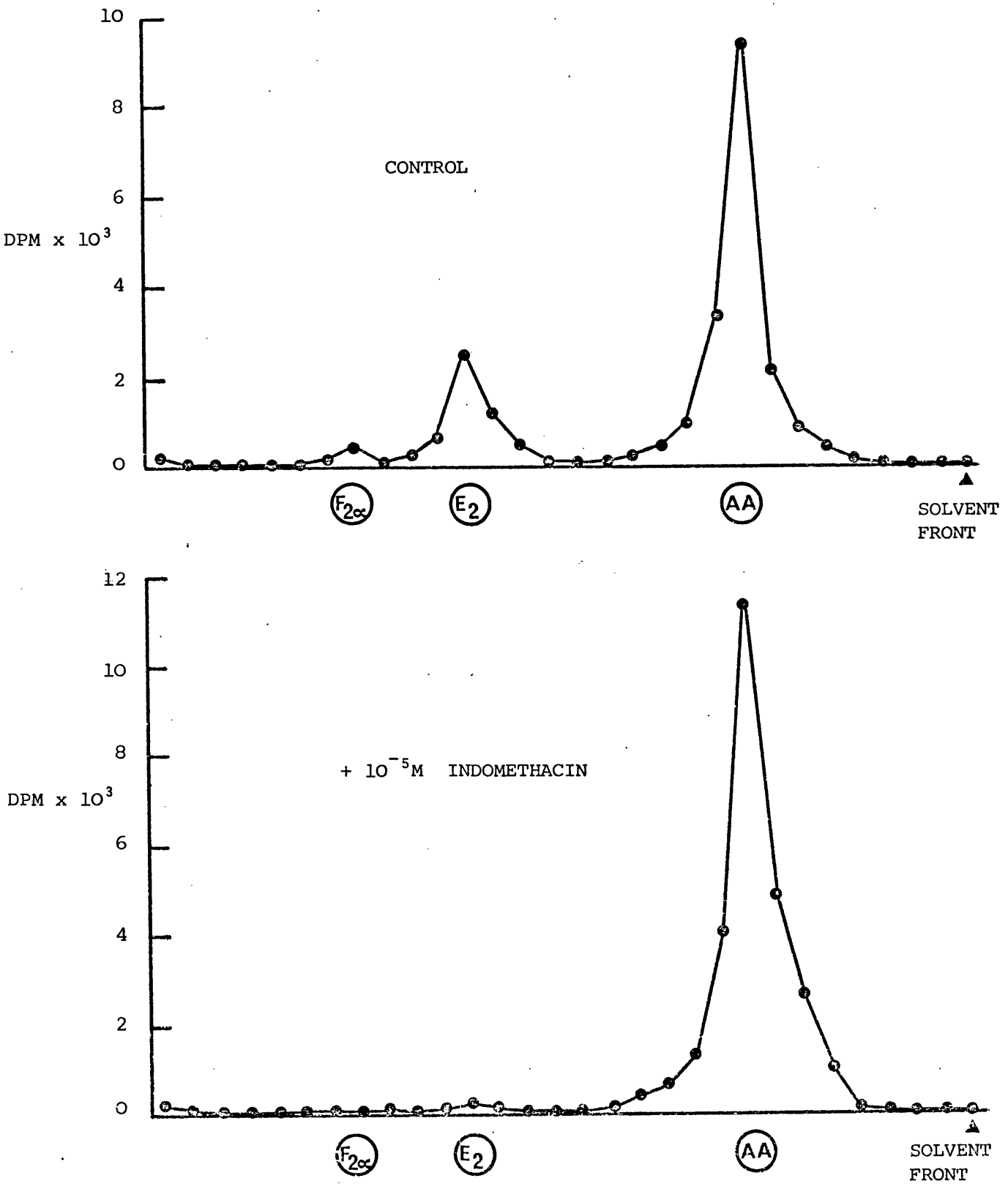
Systematic studies of existing aspirin-like drugs have been carried out using such assay systems (Ham and others, 1972; Tomlinson and others, 1972) and the correlation found between *in vitro* enzyme inhibition and known *in vivo* therapeutic activity cited as evidence in support of Vane's hypothesis. However, as discussed in section 1.11 an integral part of the hypothesis is that prostaglandin synthetase exists within an organism in multiple forms or isoenzymes, each having its own characteristic biochemical parameters and possessing a distinct pharmacological 'profile' with respect to inhibition by the aspirin-like drugs.

The inescapable conclusion to be drawn from this hypothesis is that if meaningful results are to be obtained from studies of the interaction between aspirin-like drugs and prostaglandin synthetase, the enzyme used must be derived from human tissue involved in the inflammatory reaction ("target" tissue). If such tissue is not readily available then at the very least it should be shown that the enzyme prepared from human inflammatory tissue does not differ radically in any respect from the well-studied animal preparations commonly used. When this study was initiated no such reports existed in the literature and this, in essence, forms the basis of the work presented in this chapter.

3.2 Metabolism of arachidonic acid by R.A. synovial microsomes

A microsomal fraction was prepared from human R.A. synovial tissue as described in section 2.3, incubated with ^{14}C-arachidonic acid and an ether extract of the acidified incubate separated by thin-layer chromatography (sections 2.4 and 2.5). Fig. 3.1 shows the scan obtained when 0.5 cm bands were scraped from the plate and the radioactivity in each band measured using liquid scintillation

Fig. 3.1 Metabolism of ^{14}C-arachidonic acid by human rheumatoid synovial microsomes



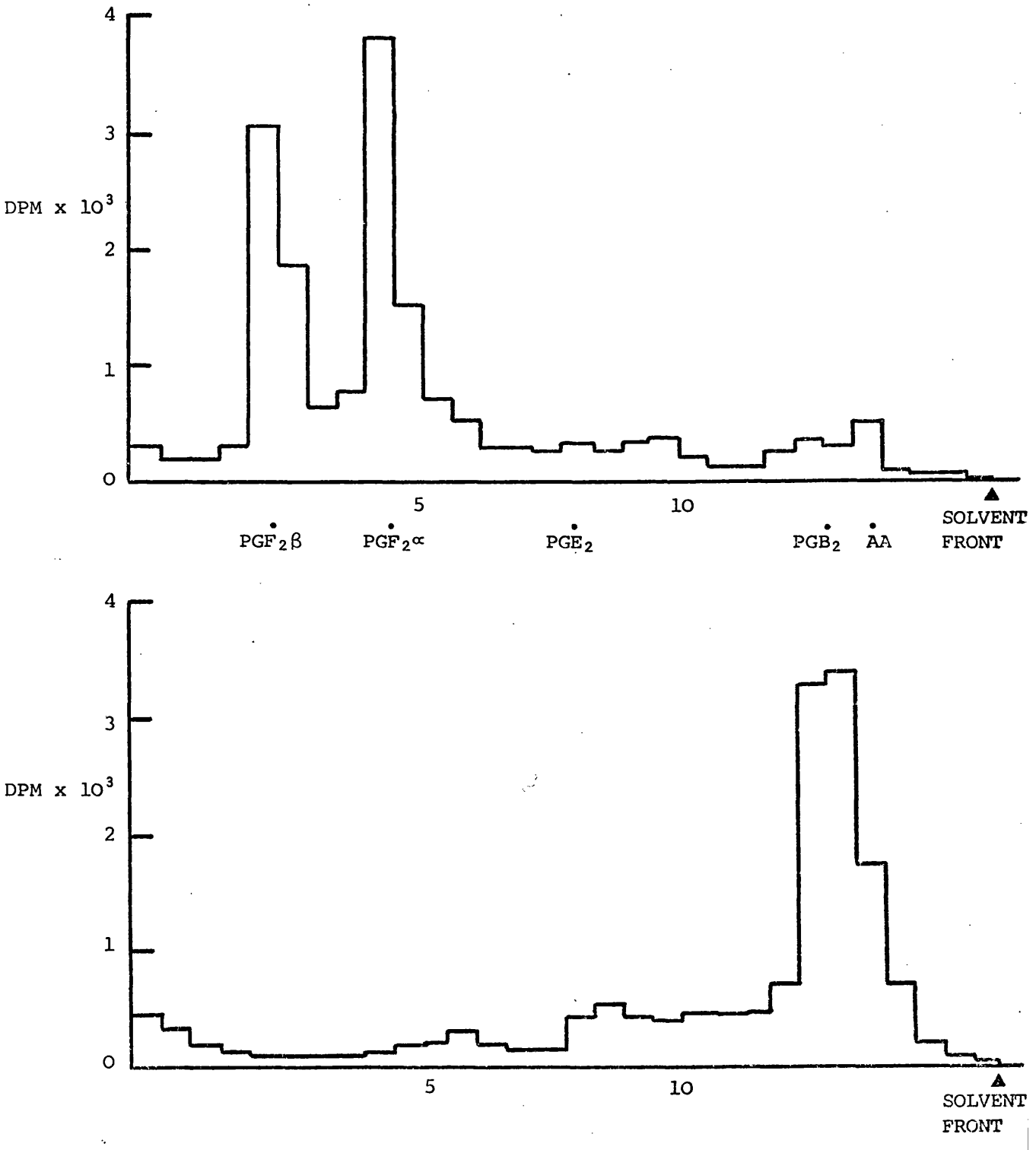
Chromatographic separation of an incubation of R.A. synovial microsomes with ^{14}C-arachidonic acid. The formation of ^{14}C-PGE_2 and ^{14}C-$PGF_2\alpha$ was abolished by the addition of indomethacin (10^{-5}M) to an identical incubate. Positions of authentic markers are shown below the scans.

counting. As can be seen the microsomal fraction from human R.A. synovial tissue is capable of producing significant metabolism of added ^{14}C-arachidonic acid; on the basis of their chromatographic mobilities the two products were tentatively identified as PGE_2 and $PGF_{2\alpha}$, constituting 21.0% and 1.4% respectively of the total radioactivity on the plate. The combined radioactivity from the areas of the plate corresponding to authentic PGE_2 , $PGF_2\alpha$ and arachidonic acid made up 95% of the total activity present and no other radioactive peaks were detected. When an identical incubate, to which had been added indomethacin (10^{-5}M), was assayed for arachidonic acid metabolism, the radioactive peaks seen in the first incubation were abolished, showing that the peaks produced during the incubation were unlikely to be chemical artefacts caused by auto-oxidation of the substrate. Furthermore as other enzyme systems likely to metabolise arachidonic acid (e.g. lipoxygenases) are not inhibited by indomethacin at this concentration this provided further tentative evidence that the radioactive peaks seen were products of the cyclo-oxygenase (i.e. prostaglandin-producing) system.

3.3 Chemical conversion of PGE_2

Further evidence for the identification of the major product of arachidonic acid metabolism as PGE_2 was sought by chemical conversion of the product to other prostaglandins, followed by chromatographic identification, as described in section 2.6. Treatment of formed ^{14}C-PGE_2 with sodium hydroxide followed by further chromatography showed that 83% of the original activity was now coincident with a standard PGB_2 marker (Fig. 3.2). Similarly treatment with sodium borohydride gave two peaks which were

Fig. 3.2 Chemical conversion of formed ^{14}C-PGE_2 with sodium borohydride or sodium hydroxide



^{14}C-PGE_2 formed from the incubation of R.A. synovial microsomes with ^{14}C-arachidonic acid was isolated by thin-layer chromatography and treated with either sodium borohydride or sodium hydroxide. Re-chromatography of the extract showed conversion to an equal mixture of $PGF_2\alpha$ and $PGF_2\beta$ upon borohydride treatment (upper scan), and conversion to PGB_2 with sodium hydroxide (lower scan).

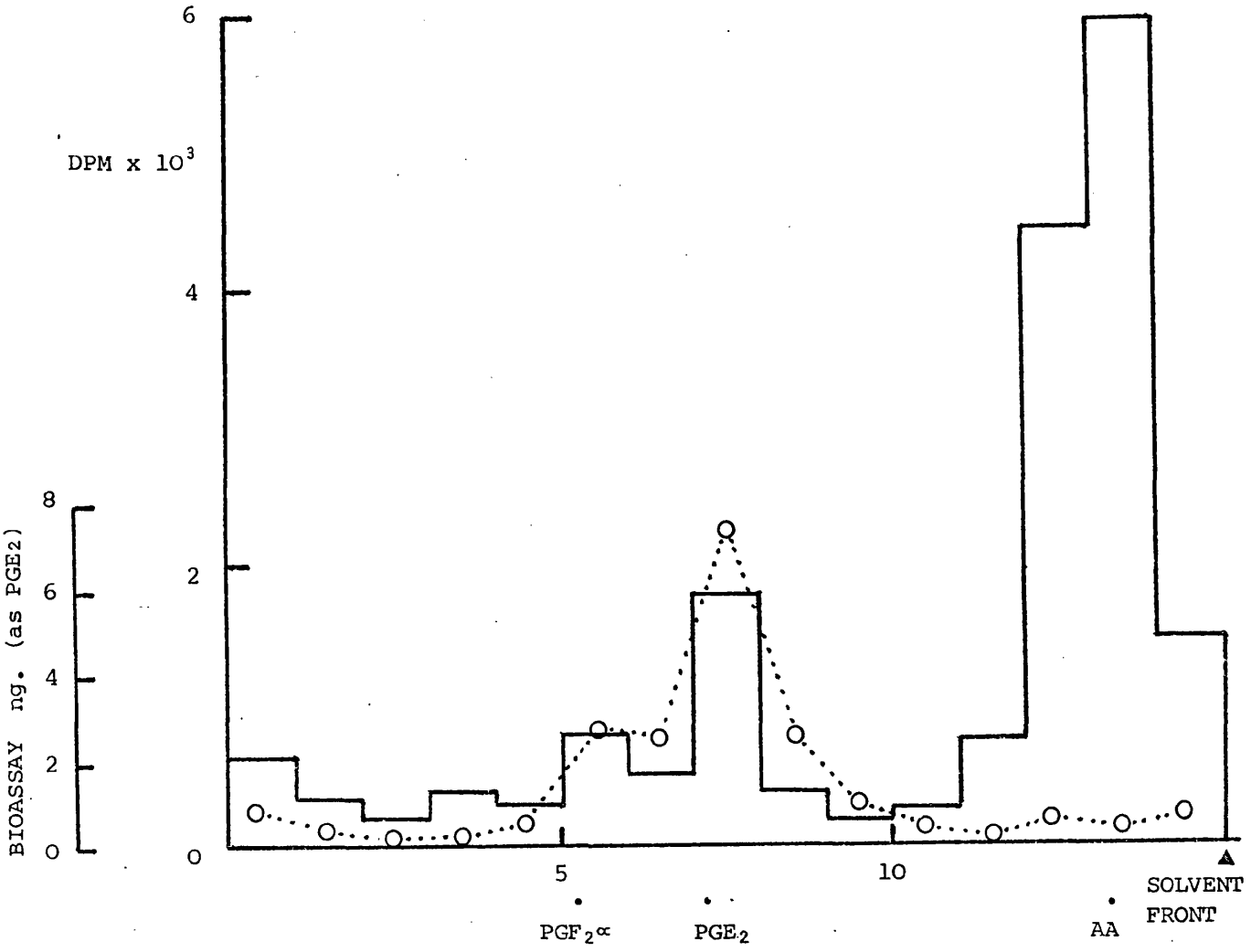
coincident with standard $PGF_{2}\alpha$ and $PGF_{2}\beta$, containing 39% and 37% of the original activity respectively (figures not corrected for procedural losses). No other significant peaks of radioactivity were found in either extract and no material remained, running in the position of PGE_2 , which was resistant to chemical conversion. Both of these chemical conversions are characteristic of PGE_2 and it is highly unlikely that any compound other than PGE_2 would behave in this manner upon treatment with sodium hydroxide or sodium borohydride. Further identification might have been achieved by measuring the increase in optical density at 278nm when a solution of PGE is treated with sodium hydroxide, though the sensitivity of this assay would probably not be adequate (A 20% conversion of the ^{14}C-arachidonic acid substrate produces ca. 100ng ^{14}C-PGE_2).

3.4 Bioassay of extracts after T.L.C.

Further confirmation of the identities of the products of arachidonic acid metabolism was sought by subjecting the ether extract of an incubate of R.A. synovial microsomes and arachidonic acid to thin-layer chromatography, followed by bioassay of 1 cm band eluates from the plate, as described in section 2.6.

When fifteen 1 cm band eluates were each bioassayed for their prostaglandin content a single major peak of activity was found (Fig. 3.3) which corresponded to the radioactive peak designated PGE_2 , and was coincident with authentic PGE_2 run in parallel. Calculation showed that the amount of PGE_2 present as measured by bioassay was in reasonably good agreement with that found from the radioactivity present, no attempt being made to correct for procedural losses. A small amount of activity was seen in the $PGF_{2}\alpha$ region and this would presumably have been more readily

Fig. 3.3 Bioassay of an extract of R.A. synovial microsomes incubated with ^{14}C-arachidonic acid after chromatographic separation.



1 cm. bands from the thin-layer separation of an extract of R.A. synovial microsomes, incubated with ^{14}C-arachidonic acid, were eluted and subjected to bioassay on a superfused rat stomach strip. The major peak of biological activity (O·····O) corresponded to the peak of radioactivity running in the position of authentic PGE_2, shown below the scan.

and selectively detected on the rat colon strip preparation.

Final confirmation of the identities of the products of arachidonic acid metabolism would depend upon identification by combined gas chromatography - mass spectrometry but facilities were not available to attempt this. However combined chromatographic, chemical and bioassay results strongly support the original tentative designations of the products as PGE_2 and $PGF_2\alpha$.

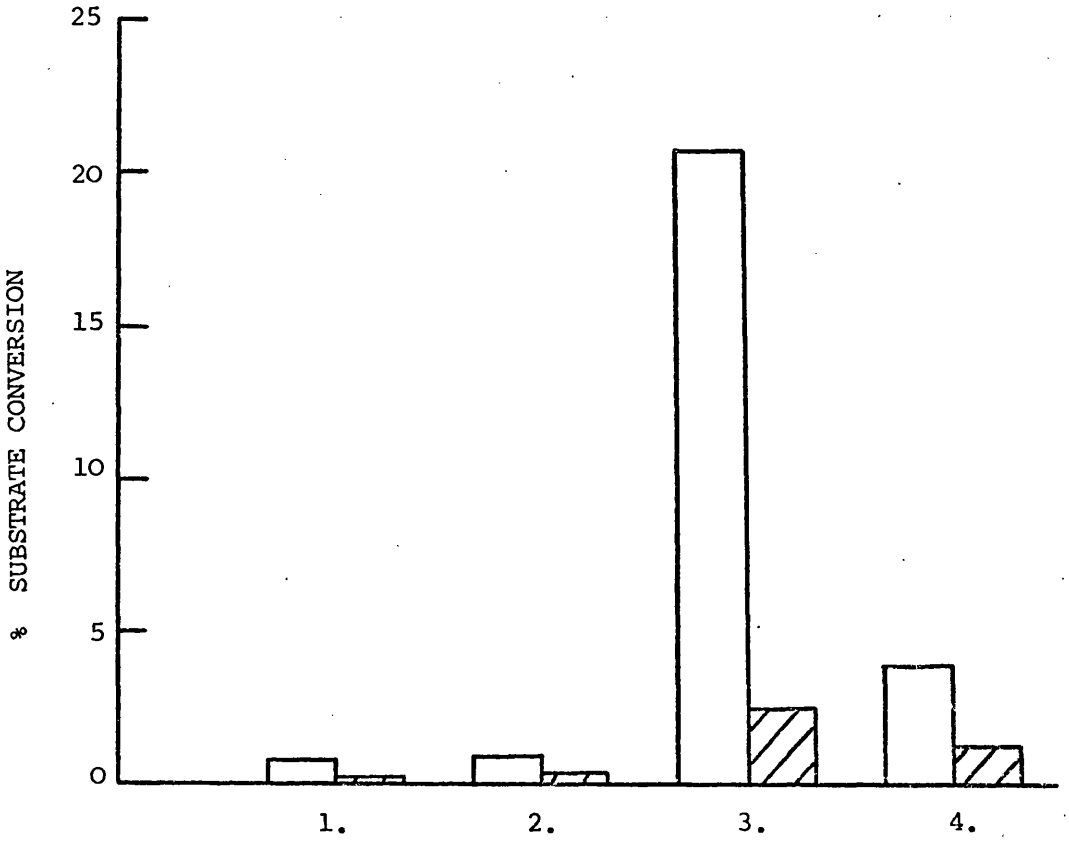
3.5 Location of PG synthetase activity

The relative amounts of PG synthetase activity present in the microsomal and high-speed supernatant fractions of R.A. synovial tissue are shown in Fig. 3.4 and confirm that, as found in other tissues studied, the activity resides in the microsomal fraction. No activity was present in the supernatant fraction as the tiny amount of activity present can also be demonstrated in a preparation of boiled microsomes. This residual 'blank' value (usually representing less than 1% substrate conversion) is probably caused by very slight streaking of the arachidonic acid region on the TLC plate and was subtracted from all subsequent values, a boiled control being assayed in each experiment for this purpose.

As found in the preliminary studies (section 2.8), using rabbit renal medulla and bovine seminal vesicle preparations, the PG synthetase activity of R.A. synovial microsomes was 'inhibited' when the high-speed supernatant fraction of the homogenate was added back to the active microsomal fraction.

It is well known that high-speed supernatant fractions from many tissues contain prostaglandin metabolising enzymes, such as 15-hydroxyprostaglandin dehydrogenase, which could explain this

Fig. 3.4 Conversion of ^{14}C-arachidonic acid to ^{14}C-PGE_2 and ^{14}C-$PGF_{2\alpha}$ human rheumatoid synovial fractions



1. Microsomal supernatant, 1.0 ml (5.2 mg protein)
2. Boiled microsomes 1.0 ml (1.8 mg protein)
3. Microsomes, 1.0 ml (1.8 mg protein).
4. Microsomes, 1.0 ml with added supernatant (1.0 ml).

□ PGE_2 ▨ $PGF_{2\alpha}$

finding. However no chromatographic evidence for the production of such metabolites was found in any incubation which contained an aliquot of the supernatant fraction and the author remains convinced that the phenomenon is due to liberation of unlabelled substrate by phospholipase activity present in the supernatant. Attempts were made to confirm this explanation by the use of mepacrine, a known phospholipase inhibitor, but these were unsuccessful as at the concentrations necessary to inhibit phospholipase activity (3×10^{-3}M), mepacrine also largely abolishes prostaglandin synthetase activity; perhaps a more 'selective' inhibitor could have resolved the issue.

Clearly it is desirable to use the microsomal fraction of the tissue as the source of PG synthetase and the findings explain the failure of attempts to demonstrate PG synthetase activity in a cell-free homogenate of rheumatoid synovial tissue.

3.6 PG metabolising activity

The possibility that the microsomal fraction might contain prostaglandin metabolising activity was investigated by adding ^{3}H-PGE_2 and ^{3}H-$PGF_{2\alpha}$ as substrate, in place of ^{14}C-arachidonic acid, to separate microsomal incubates. These were then taken through exactly the same incubation, extraction and incubation procedures as for the arachidonate incubates. Chromatography showed that 94% and 97% of the radioactivity present corresponded to authentic PGE_2 and $PGF_{2\alpha}$ respectively; no other peaks of radioactivity were detected through likely metabolites (15-oxo or 13,14-dihydro-15-oxo) would have been readily detected in the chromatographic system used. This suggests that under the incubation conditions described the microsomal preparation from R.A. synovial tissue contains no prostaglandin metabolising activity. It is necessary to demonstrate

this lack of metabolising activity as the aspirin-like drugs are known to inhibit 15-hydroxyprostaglandin dehydrogenase (Hansen, 1972); erroneous results would be obtained if the effect of aspirin-like drugs was studied in a system in which both PG synthetase and PG metabolising activity was present.

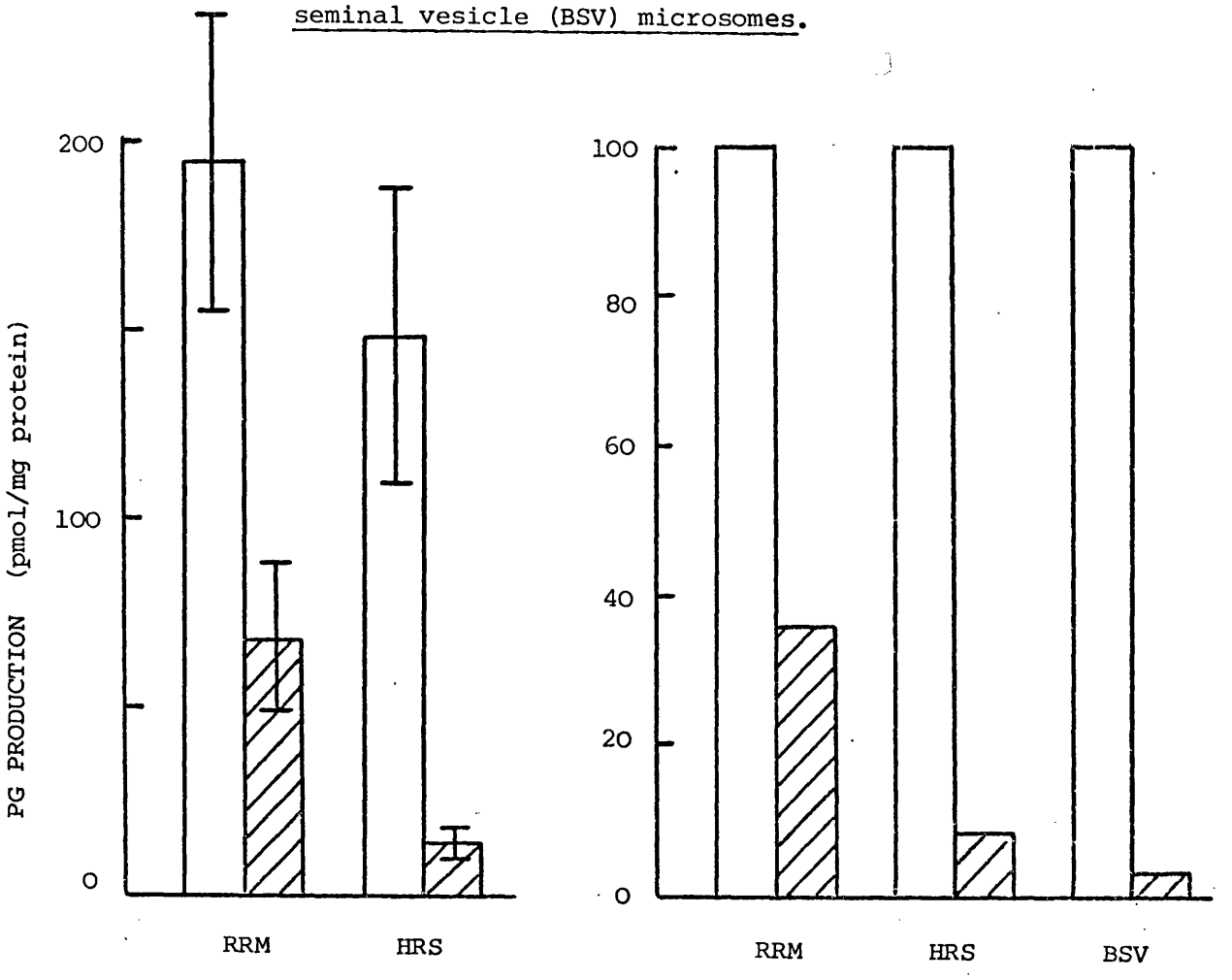
3.7 Level of PG synthetase in R.A. synovial microsomes

There is no internationally agreed unit for prostaglandin synthetase activity so that it is difficult to report absolute levels of the enzyme present in a tissue or tissue preparation. As shown by Christ and van Dorp (1972) the level of PG synthetase activity varies considerably between different tissues; when incubated with all-cis-8,11,14-eicosatrienoic acid most tissue homogenates produced only a low yield of PGE_1 (at most only a few percent conversion of substrate) though moderate yields (5-10%) were found for homogenates of lung and renal medullary tissue and high yields (> 20%) only for sheep vesicular glands, rabbit renal medulla and the urinary bladder of the frog.

Fig. 3.5A shows the comparative production of prostaglandins by microsomal preparations of rabbit renal medulla and R.A. synovial microsomes, assayed under identical conditions. Using the above criteria it must be concluded that R.A. synovial tissue is a good source of prostaglandin synthetase.

Fig. 3.5B shows that under identical incubation conditions microsomal preparations from rabbit renal medulla, R.A. synovia and bovine seminal vesicles produce different ratios of PGE_2 to $PGF_2\alpha$, the values being ca. 3:1, 10:1 and 20:1 respectively. The predominance of PGE_2 production over $PGF_2\alpha$ in all three preparations

Fig. 3.5 Production of prostaglandins by human rheumatoid synovial (HRS) and rabbit renal medulla (RRM) microsomes, and relative proportions of ^{14}C-PGE_2 and ^{14}C-$PGF_{2\alpha}$ produced by HRS, RRM and bovine seminal vesicle (BSV) microsomes.



(A) (B)

(A) PGE_2 □ and $PGF_{2\alpha}$ ▨ were formed by incubation of microsomes in 0.25M sucrose/0.1M tris-acetate, at pH 8.0 with ^{14}C-arachidonic acid (100nCi) for 1 hr. at 37°c. Mean amounts of PG's produced are shown with the standard error of the mean for each (RRM : 3 preparations; HRS : 8 preparations).

(B) Relative proportions of PGE_2 □ and $PGF_{2\alpha}$ ▨ formed by rabbit renal medulla (RRM), human rheumatoid synovial (HRS) and bovine seminal vesicle (BSV) microsomes under identical incubation conditions. Production of PGE_2 was taken as 100% for each preparation.

is expected as the presence of reduced glutathione in the incubation mixture is known to favour PGE_2 production at the expense of $PGF_{2\alpha}$ (Lands, Lee and Smith, 1971).

Throughout these studies yields of $PGF_{2\alpha}$ from R.A. synovial microsomes were generally low (<2% substrate conversion) under the incubation conditions employed and most of the values of PG synthetase activity presented have been calculated on the basis of PGE_2 production. However in a few very active preparations the yields of $PGF_{2\alpha}$ were somewhat greater (2-5% substrate conversion) and in these experiments reliable and accurate figures could be obtained for the production (and often inhibition by drugs) of both PGE_2 and $PGF_{2\alpha}$. In such cases good agreement was obtained between results calculated on the basis of either PGE_2 or $PGF_{2\alpha}$ production.

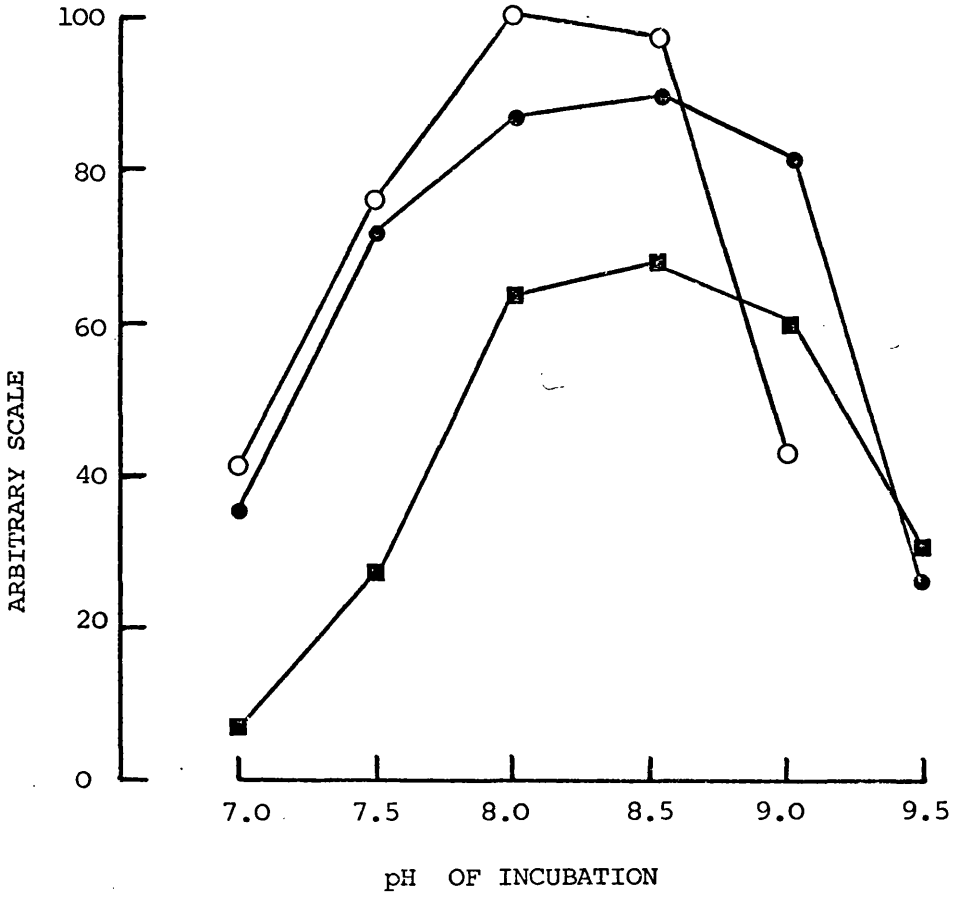
3.8 pH profiles

The pH at which an enzyme exhibits its optimum activity is a fundamental property of that enzyme. In view of the hypothesis discussed earlier that PG synthetase may exist as isoenzymes within different tissues it was of interest to compare the pH profile of the enzyme from R.A. synovium with that of rabbit renal medulla and bovine seminal vesicle. When incubations were carried out over the pH range 7.0 - 9.5 all three preparations produced a similar profile (Fig. 3.6) with pH optima in the region 8.0 - 8.5; it was not possible to distinguish between the three preparations on the basis of their pH profiles, through perhaps one might not expect isoenzymes to have markedly different pH optima.

3.9 Cofactor requirements

Although PG synthetase activity is found in the microsomal

Fig. 3.6 pH profiles of microsomal PG synthetase activity



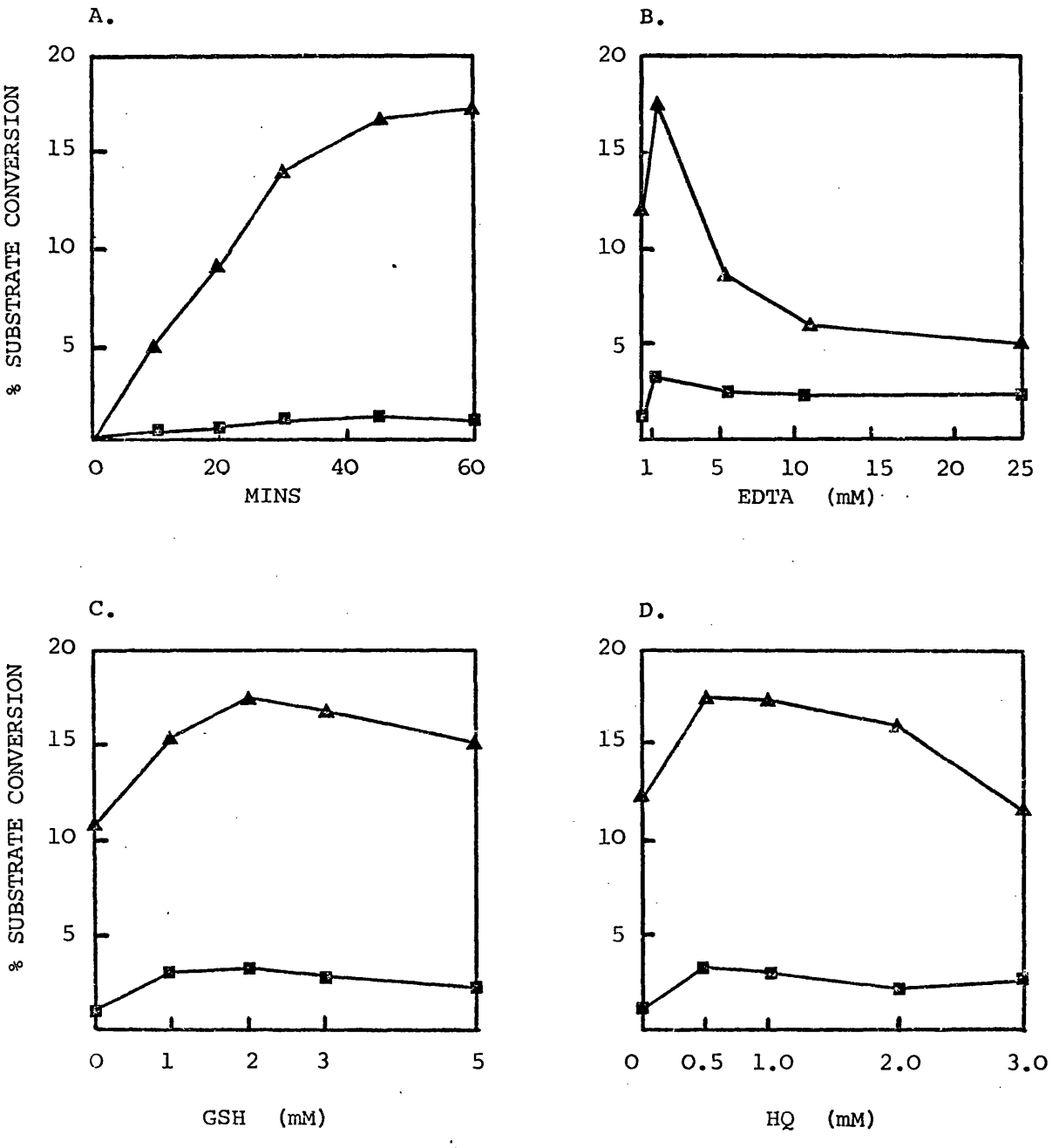
Production of ^{14}C-PGE$_2$ from ^{14}C-arachidonic acid (100 nCi/ incubation) by human rheumatoid synovium (O——O), rabbit renal medulla (●——●) and bovine seminal vesicle (■——■) microsomes over a pH range. Incubations were carried out in 0.25M sucrose/0.1M tris-acetate buffer for 1 hr. at 37°c in a volume of 2.0 ml.

fraction of cells, heat-stable cofactors from the cytoplasmic fraction are necessary for appreciable activity to be demonstrated in vitro. Reduced glutathione (GSH) has been found to act as a suitable cofactor, together with a phenolic compound such as adrenaline or hydroquinone (HQ). In addition ethylenediamine tetracetic acid (EDTA) is often included in incubations, both to remove metal ions which might have an inhibitory effect on the enzyme (Nugteren, Beerthuis and van Dorp, 1966) and also to inhibit any phospholipase activity which might give spurious results due to liberation of endogenous substrate(s), phospholipase being a calcium-dependant enzyme. The optimum concentrations of these three compounds to produce maximum PG synthetase activity were therefore investigated, the results being shown in Fig. 3.7. GSH and HQ both enhance PG synthesis at the lower concentrations studied, optimal values being 2mM and 0.5mM respectively. Concentrations higher than these produce no further stimulation of enzyme activity and may in fact be inhibitory to the enzyme. The addition of 1mM EDTA enhances enzyme activity by approximately 50%, though higher concentrations are markedly inhibitory. As can be seen from Fig. 3.7 production of $PGF_2\alpha$ tended to parallel production of PGE_2 and there was no evidence that either GSH or HQ could enhance production of one prostaglandin at the expense of the other.

3.10 Kinetic studies

The time course of PG production was investigated in order to determine an optimum incubation time for the enzyme and also to measure the time over which the formation of products was linear. Incubations of R.A. synovial microsomes with ^{14}C-arichidonic acid were carried out as described in section 2.4 and the reaction terminated at various times by the addition of 2M citric acid solution (0.25ml). The amounts of PG's produced in each sample were then

Fig. 3.7 Time course of PG production by R.A. synovial microsomes and influence of co-factor concentrations



A. R.A. synovial microsomes were incubated with ^{14}C-arachidonic acid for varying times and the amounts of PGE_2 (▲——▲) and $PGF_{2\alpha}$ (■——■) measured as described in the text. PG production was linearly related to time for the first 30 minutes of the incubation.

B. C. and D.

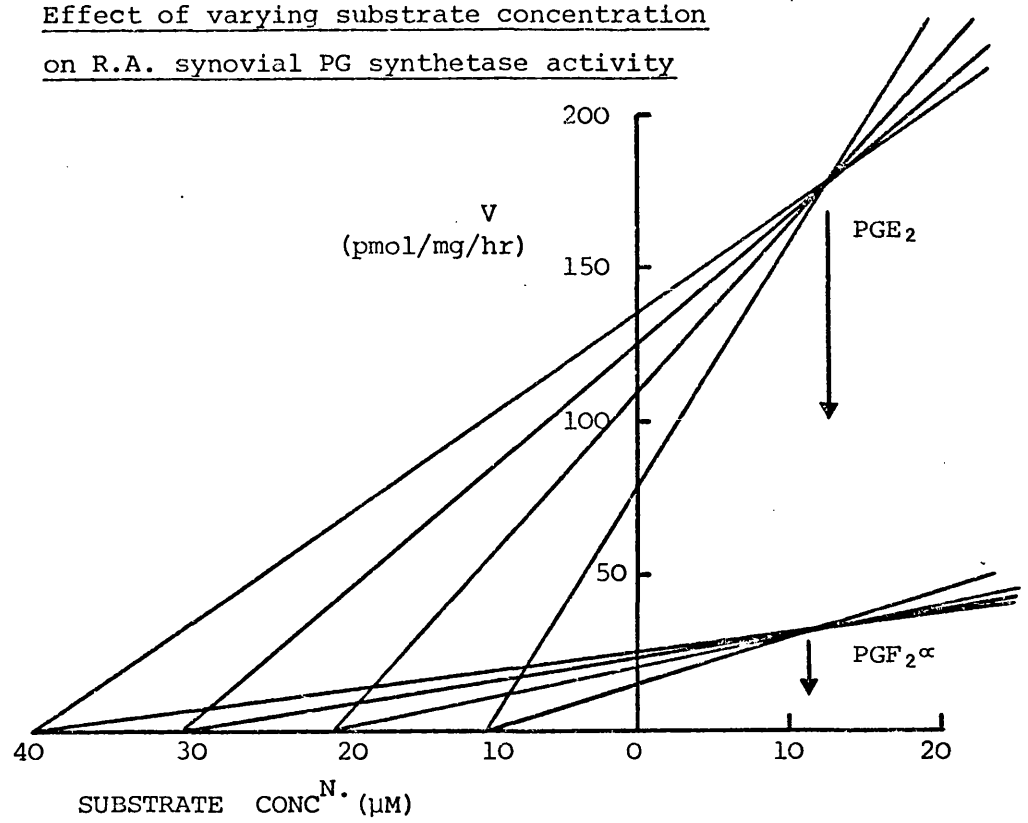
Varying the co-factor concentrations showed the optimum enzyme activity was found with 1mM EDTA, 2mM GSH and 0.5mM HQ.

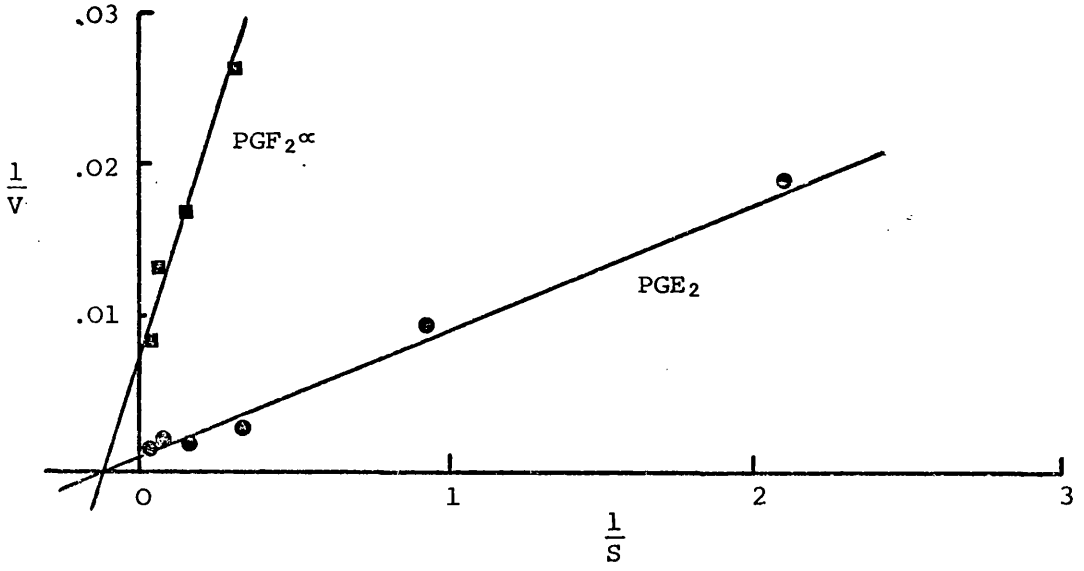
determined as described (section 2.5). The results (Fig. 3.7a) show that production of both PGE_2 and $PGF_{2\alpha}$ is linear with respect to time for the first 30 minutes of the incubation, the initial rates being 35pmol/mg/min and 4.4pmol/mg/min respectively in the preparation shown. A further increase in PG production occurs over the next 30 minutes through this is no longer linearly related to the time of incubation, representing an additional 21% production over the 30 minute value. For determination of the Michaelis constant (Km), described below, an incubation time of 30 minutes was therefore chosen, though one hour was used in all other experiments.

Another fundamental property of an enzyme is its Km value, i.e. the substrate concentration producing half maximum velocity. This was determined for R.A. synovial PG synthetase by varying the substrate concentration over the range 0.4-40μM. As the amount of ^{14}C-arachidonic acid needed to achieve this concentration would have been excessive the amount of labelled arachidonic acid in each incubation was kept constant (100 nCi) and the total concentration desired achieved by the addition of unlabelled arachidonic acid; multiplication of the percentage conversion of the labelled substrate to PGE_2 by the 'total' substrate concentration in each incubation gave the velocity of the reaction at each substrate concentration.

When this was done the enzyme exhibited typical saturation kinetics and the results are shown in Fig. 3.8. In the upper plot the results are in the form of a 'direct linear plot' (Eisenthal and Cornish-Bowden, 1974) and yield values for the Km of 12μM and 11μM, calculated on the basis of PGE_2 and $PGF_{2\alpha}$ production respectively. This finding is in agreement with the generally accepted belief that synthesis of PGE_2 and $PGF_{2\alpha}$ proceeds via a common intermediate (Samuelsson, 1972).

Fig. 3.8 Effect of varying substrate concentration on R.A. synovial PG synthetase activity





PG production by R.A. synovial microsomes was measured at substrate concentration from 0.4-40μM. The results are presented as a direct linear plot (Eisenthal and Cornish-Bowden, 1974) and as a conventional double-reciprocal plot. Both yield a K_m value of approximately 12μM.

In the lower plot of Fig. 3.8 the results of varying the substrate concentration are shown as a conventional double-reciprocal (Lineweaver-Burke) plot; as can be seen the lines obtained for PGE_2 and $PGF_{2\alpha}$ production have a common intercept on the -x axis, yielding a Km of 12.5μM.

3.11 Discussion

Prostaglandin synthetase activity has been found in virtually every mammalian tissue studied, as well as in tissues from other vertebrate and invertebrate species (Christ and van Dorp, 1972). Numerous experiments using tissue homogenates, isolated organs or whole animals have confirmed that prostaglandin synthetase may indeed be considered a ubiquitous enzyme (see Flower, 1974). Using tissue culture techniques it has been conclusively shown (Levine, 1973; Robinson and McGuire, 1975) that fragments of R.A. synovium are capable of significant production of prostaglandins *in vitro*. Furthermore, human R.A. synovial fluid contains readily measured amounts of prostaglandins, and the levels are several-fold higher than those found in normal fluids (Levine 1973; Higgs and others, 1974).

In view of the above results it is perhaps not surprising therefore to find that R.A. synovium is capable of prostaglandin biosynthesis *in vitro*. As in all other tissues studied, the enzyme activity is associated with the high-speed particulate, 'microsomal', fraction. The term 'microsomal fraction' however tells us nothing about the cellular location of the enzyme, and the use of such a term has been criticised (De Duve, 1971). No attempt was made in the present work to fractionate the crude microsomal preparation in order to locate the site of PG biosynthesis within the cell, though this has been achieved by others (Bohman and Larsson, 1975) studying

membrane fractions from rabbit renal medulla. Using sucrose gradients 3 membrane fractions were isolated from a 'microsomal' preparation and the subcellular origin of the membranes determined by electron microscopy and marker enzyme content; PG synthetase activity was also measured in each fraction using a radiometric technique similar to that used in the present work. Fraction II, the cytomembrane fraction, (consisting of closed vesicles of both smooth-surfaced and ribosome-studded membranes) contained the majority of the PG synthetase activity, though some activity was detectable in fraction I (a plasma membrane fraction) and fraction III (mitochondrial fraction). The authors concluded that PG synthetase is located in the endoplasmic reticulum of renal medullary cells.

Having demonstrated that R.A. synovial microsomes were capable of producing significant metabolism of added ^{14}C-arachidonic acid it was essential to characterise the products before proceeding with further studies as enzyme systems other than PG synthetase are able to metabolise polyunsaturated fatty acids (e.g. lipoxygenases) and identification of products on the basis of mobilities in a single solvent system, when compared with authentic marker compounds, would be most unsafe. The combined use of three different chromatographic solvent systems, chemical conversions of the principal product to other products followed by further chromatography, and bioassay of TLC eluates demonstrated beyond reasonable doubt that the original tentative identifications of the products of arachidonic acid metabolism as PGE_2 and $PGF_{2\alpha}$ were justified. Absolute confirmation would require the use of combined GC/MS facilities and these were not available to the author. However PGE_2 and $PGF_{2\alpha}$ are designated as such throughout this work on the basis of the above criteria in order to avoid the use of the cumbersome 'PGE_2-like' etc.

When the high-speed supernatant of a homogenate of R.A. synovium was added back to an active microsomal fraction, an inhibition of the conversion of ^{14}C-arachidonic acid to ^{14}C-PGE_2 and ^{14}C-$PGF_{2\alpha}$ was seen, confirming the preliminary studies with rabbit renal medulla and bovine seminal vesicles reported in section 2.8. If this phenomenon was due to the presence of a natural inhibitor of PG biosynthesis in the cytoplasmic fraction of the cell this would be of considerable importance to an understanding of the cellular control of PG synthesis and would certainly warrant further study. However results from other studies suggest that this is unlikely to be the case; Pace-Asciak and Wolfe (1970) studying prostaglandin synthesis in rat stomach homogenates found that a considerable dilution of the added substrate by endogenous arachidonic acid took place during incubation and that prostaglandins were formed from both substrates. Had these authors measured only the tritium-labelled products without taking into account unlabelled products formed from endogenous precursor they would certainly have observed an apparent 'inhibition' of PG synthesis in a cell-free homogenate when compared with a microsomal fraction from the same preparation.

Results obtained by Samuelsson (1970) lend further support to these findings; when a microsomal fraction of sheep vesicular gland was incubated with a labelled phospholipid (containing ^{14}C-dihomo-γ-linolenic acid in the 2-position), ^{14}C-PGE_1 was produced in only trace amounts. Addition of a high-speed supernatant fraction from rat liver, however, resulted in the production of large amounts of ^{14}C-PGE_1, showing that the supernatant fraction contained phospholipase activity capable of liberating polyunsaturated fatty acid(s) from phospholipid. As phospholipids are ubiquitous components of such sub-cellular fractions it is not unreasonable to suppose that any

high-speed supernatant is capable of liberating prostaglandin precursor(s) from phospholipid.

Since calcium ions are necessary for optimum phospholipase A activity (Newkirk and Waite, 1971) the inclusion of EDTA in the buffer used in the present studies might be expected to reduce, if not abolish this activity; perhaps at the concentration used (1mM) some residual phospholipase activity remains, sufficient to produce the effects described above. It is of interest to note that the pH optimum for PG synthesis at which the incubations were carried out (pH 8.0) is also the optimum for phospholipase A_2 activity (Bjørnstad, 1966; de Haas and others, 1968). As shown in section 3.9, higher concentrations of EDTA markedly inhibited PG synthetase activity.

Confirmation that this 'inhibitory' effect of the cytoplasmic fraction of R.A. synovial tissue was due to release of endogenous substrate(s) was sought with the use of mepacrine, a known phospholipase inhibitor (Kunze and Vogt, 1971); however at the concentration used (3×10^{-3}M) prostaglandin biosynthesis was virtually abolished and the values obtained were too low for accurate quantitation. A more specific phospholipase inhibitor would possibly have provided the answer.

The finding that R.A. synovial microsomes exhibit no prostaglandin-metabolising activity under the standard incubation conditions used was not surprising, as these metabolising enzymes have been shown in many other tissues to be located in the cytoplasmic fraction and to be NAD dependent. (Anggard and Samuelsson, 1966). Nevertheless it was necessary to demonstrate this lack of PG metabolism under the chosen incubation conditions as the later studies of the inhibition of PG synthesis by aspirin-like drugs could have led to erroneous results

in the presence of PG metabolising activity, as some aspirin-like drugs are known to inhibit 15-hydroxyprostaglandin dehydrogenase (Hansen, 1972).

When assayed under identical conditions the microsomal fractions from R.A. synovial tissue and rabbit renal medullae were found to contain similar levels of PG synthetase activity. As rabbit renal medulla is considered to be a good source of PG synthetase (Christ and Van Dorp, 1972), R.A. synovium must also be regarded as a tissue containing appreciable activity. Perhaps in the future an 'international unit' of PG synthetase activity will be defined in order to facilitate more objective comparisons between different tissues or between the findings of different workers.

When incubated over a pH range PG synthetase prepared from R.A. synovium was found to exhibit optimum activity at pH 8.0. Most other tissues studied have been found to exhibit optimum PG synthetase activity at pH 8.0-8.2, though the enzyme from rabbit renal medulla has been reported to be maximally active at pH 7.5 (Rose and Collins, 1974; Blackwell, Flower and Vane, 1975). The latter authors also reported that the enzyme was very sensitive to small changes in pH, a pH change of only 0.2 from the optimum resulting in a decrease in product formation of 45-55%. This degree of sensitivity was not observed in the present work in any of the preparations studied and is possibly a reflection of the substrate concentrations used. In the present work a low (1μM), 'physiological' substrate concentration was used whereas in the work described above a concentration of 2mM was employed. At these extremely high substrate levels minor products of arachidonic acid metabolism *in vitro* may be exerting pH-dependent inhibitory effects on the enzyme which would be quite insignificant *in vivo*. Speculation that the high sensitivity of the

kidney enzyme to changes in pH has a physiological significance must therefore be viewed with some scepticism.

Other studies of PG synthetase from rabbit renal medulla are at variance with the results of Blackwell, Flower and Vane (1975); using a substrate concentration of 60μM, Tai, Tai and Hollander (1976) found that the enzyme exhibited a virtually flat pH profile from pH 7.0 to pH 8.5 similar to that reported in the present work. Similarly Schwartzman, Gafni and Raz (1976) reported a broad pH profile for both a homogenate and a microsomal preparation with optimum activity at pH 8.4, using a low (3.3μM) substrate concentration.

The cofactor requirements of bovine seminal vesicle PG synthetase are well documented (Yoshimoto, Ito and Tomita, 1970; Lands, Lee and Smith, 1971; Tomlinson and others, 1972). Reported optimum concentrations were in the range 1.0-2.0mM for GSH and 0.2-0.5mM for HQ. In the present work the optimum requirement of PG synthetase, prepared from R.A. synovium, for GSH and HQ was found to be 2mM and 0.5mM respectively, in good agreement with the above. Similar findings have been reported for the enzyme prepared from rabbit renal medulla (Tai, Tai and Hollander, 1976), sheep seminal vesicular gland (Wallach and Daniels, 1971), rat skin (Kingston and Greaves, 1976), human skin (Ziboh, 1973) and even the reproductive tract of the male house cricket (Destephano, Brady and Woodall, 1976).

In the present studies the inclusion of EDTA (1mM) in the incubation buffer was found to stimulate PG synthesis though higher concentrations were markedly inhibitory. Ziboh (1973) has shown that EDTA enhances PG synthesis by human skin subcellular fractions and suggested that this may be due, in part at least, to chelation of heavy metal ions known to be inhibitory to the enzyme (Nugteren,

Beerthuis and van Dorp, 1966; Deby, Bacq and Simon, 1973). Hamberg (1969) has recommended the inclusion of EDTA at a concentration of 20mM in incubations involving whole tissue extracts. Such a concentration, using the microsomal preparations used in the present work, is markedly inhibitory (Fig. 3.7); as discussed previously part of the apparent stimulatory effect may be due to inhibition of endogenous phospholipase activity.

When the time course of prostaglandin production by R.A. synovial microsomes was studied, synthesis was found to proceed linearly for the first 30 minutes of the incubation but to decrease proportionally thereafter. Values in the literature for the time at which PG production ceases to be linearly related to the time of incubation vary greatly, and probably reflect the very different conditions, particularly the substrate concentrations used, under which the various experiments have been carried out. Using bovine seminal vesicle microsomes, Yoshimoto, Ito and Tomita (1970) found that synthesis proceeded linearly only for the first 90 seconds of incubation, whereas Kingston and Greaves (1976), using rat skin microsomes, found PG production to be directly proportional to the time of incubation for the first 20 minutes. As discussed by Wallach and Daniels (1971), who reported a time of 6 minutes for sheep seminal vesicle microsomes, the enzyme may be inhibited by one or more products of the reaction and the effect is markedly substrate dependent; removal of the enzyme from the incubation mixture by centrifugation, followed by addition of fresh substrate, allowed the reaction to proceed at its original rate, showing that the postulated inhibitor produced a competitive inhibition of the enzyme rather than a destruction of the catalytic site.

Incubation of R.A. synovial microsomes with varying concentrations of arachidonic acid over the range 0.4-40μM showed that PG synthetase exhibited typical Michaelis-Menten kinetics; the use of either a direct linear plot or a conventional double-reciprocal plot gave a Km value of approximately 12μM, calculated on the basis of either PGE_2 or $PGF_{2\alpha}$ formation. The finding that the Km value is the same when calculated on the basis of either product of arachidonic acid metabolism is consistent with the belief that synthesis of PGE_2 and $PGF_{2\alpha}$ proceeds via the common intermediate, PGH_2 (Samuelsson, 1972).

Reported values in the literature for the Km's of various PG synthetase preparations vary greatly and large discrepancies exist between the values obtained using the same tissue for the preparation of the enzyme. Thus, whilst values of 8.3μM and 10μM have been reported for the Km of rabbit renal medulla PG synthetase (Tai, Tai and Hollander, 1976; Schwartzman, Grafni and Raz, 1976), Blackwell, Flower and Vane (1975) reported an optimum substrate concentration for the same enzyme of 2mM, implying a Km of approximately 1mM. In view of such large variations in reported values it is clear that comparisons between different tissues studied in different laboratories are dubious and unlikely to yield meaningful results unless the conditions employed are standardised.

In most tissues the levels of free polyunsaturated fatty acids are very low, typically 10-50 ng/g. (Brocklehurst and Dawson, 1974). Control of PG biosynthesis is thought to be exerted by release of polyunsaturated fatty acid(s) from membrane phospholipid by phospholipase A_2 activity (Samuelsson, 1972; Flower and Blackwell, 1976), and there is no evidence to suggest that conversion of precursor to

prostaglandins is the rate-limiting step in the sequence. The present author is unable to see the justification for the use of the high substrate concentrations (1-2mM) used by some workers for *in vitro* studies as the enzyme could not possibly find itself working under such 'forced' conditions *in vivo.* Throughout the present work a 'realistic' substrate concentration of approximately 1μM was used for *in vitro* studies, well below the measured Km of 12μM.

In summary, a study of the basic biochemical properties of PG synthetase prepared from R.A. synovial tissue showed no noteworthy differences between this enzyme and the well-studied enzymes prepared from several animal tissues. As one of the tenets of Vane's hypothesis is that each isoenzyme of PG synthetase exhibits a characteristic pharmacological 'profile' in respect of inhibition by aspirin-like drugs, the interaction between this group of drugs and R.A. synovial PG synthetase was studied in some detail. These results are presented in the following chapter.

CHAPTER FOUR

THE INHIBITION OF RHEUMATOID SYNOVIAL PG SYNTHETASE

4.1 Introduction

The reports of Vane (1971), Smith and Willis (1971) and Ferreira, Moncada and Vane (1971) that aspirin-like drugs inhibited biosynthesis of prostaglandins provided for the first time a plausible hypothesis to explain the mode of action of this group of compounds. These initial results were soon confirmed in many other systems, both *in vivo* and *in vitro*, and inhibition of PG biosynthesis shown to be a property peculiar to aspirin-like drugs since many other pharmacologically active compounds are inactive against PG synthetase, even in concentrations of up to 5mM (Flower, 1974).

Prostaglandins exhibit certain inflammatory properties which suggest they may be implicated in various aspects of the inflammatory process in general, and in rheumatoid arthritis in particular (sections 1.8 and 1.9 respectively). As the aspirin-like drugs form the basis of the clinical treatment of chronic inflammatory diseases such as rheumatoid arthritis it is clearly of relevance to study the interaction between this class of drugs and PG synthetase derived from tissue involved in the disease process.

The results presented in this chapter concern principally the *in vitro* inhibition of R.A. synovial PG synthetase by aspirin-like drugs, though the effect of other antirheumatic drugs such as penicillamine and the corticosteroids has also been studied. Copper is known to influence PG biosynthesis (Lee and Lands, 1972; Maddox 1973); the interaction between copper ions and synovial PG synthetase was studied, as copper chelates of the aspirin-like drugs have been proposed as potentially valuable anti-inflammatory agents (Sorenson, 1976). Finally the therapy which patients were receiving at the time of operation was found to markedly influence subsequent microsomal

PG synthetase activity and these results are discussed in relation to the in vitro studies.

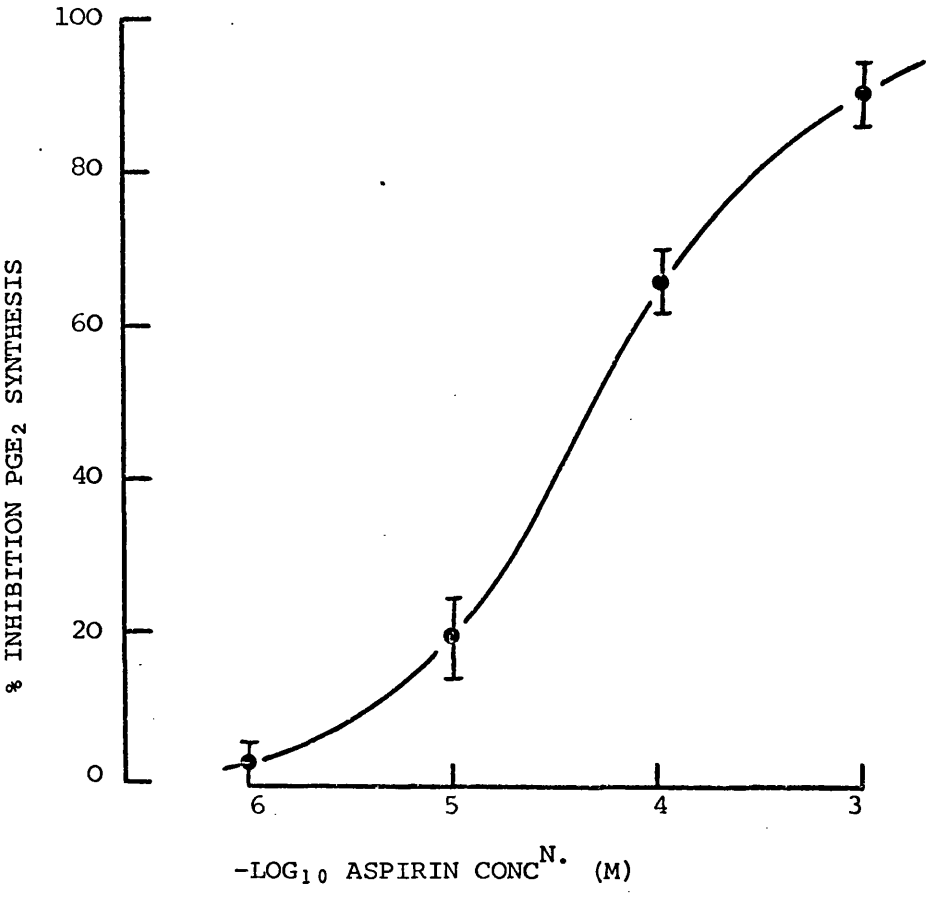
4.2 In vitro inhibition by aspirin-like drugs

The aspirin-like drugs used in these studies were the generous gifts of the manufacturing companies and are listed, together with their trade names in section 2.2. Solutions of these drugs were always made up freshly and used within 30 minutes of preparation. Usually a stock solution of the drug (10^{-2}M or 10^{-3}) was prepared in the 0.1M tris acetate buffer, pH 8.0 with the aid of a small amount (0.5ml in 25ml) of absolute alcohol, such that the concentration of ethanol never exceeded 0.2% in any incubation. (Levels of ethanol up to 2.5% were shown to have no effect on the enzyme activity). Appropriate aliquots were added to suspensions of the microsomal PG synthetase preparation and the volume made up to 1.9ml. with the tris-acetate buffer used for the incubation (section 2.4). After a 5 minute incubation of the enzyme with the drug the radioactive substrate was added and the incubation carried out as described previously.

A comparison of the amounts of ^{14}C-prostaglandins produced in incubations containing various concentrations of a drug with the amounts produced in control incubations gave a dose-response curve from which IC_{50} values (the concentration of a drug necessary to reduce PG production to 50% of control values) could be calculated (graphically). In all these studies aspirin was assayed in each experiment as a 'standard' compound and relative potencies expressed on a molar basis, taking aspirin as unity.

Fig. 4.1 shows a composite dose-response curve obtained for aspirin for nine consecutive preparations of R.A. synovial PG synthetase. The mean IC_{50} value was 45μM and the range observed

Fig. 4.1 Inhibition of R.A. synovial PG synthetase by aspirin in vitro



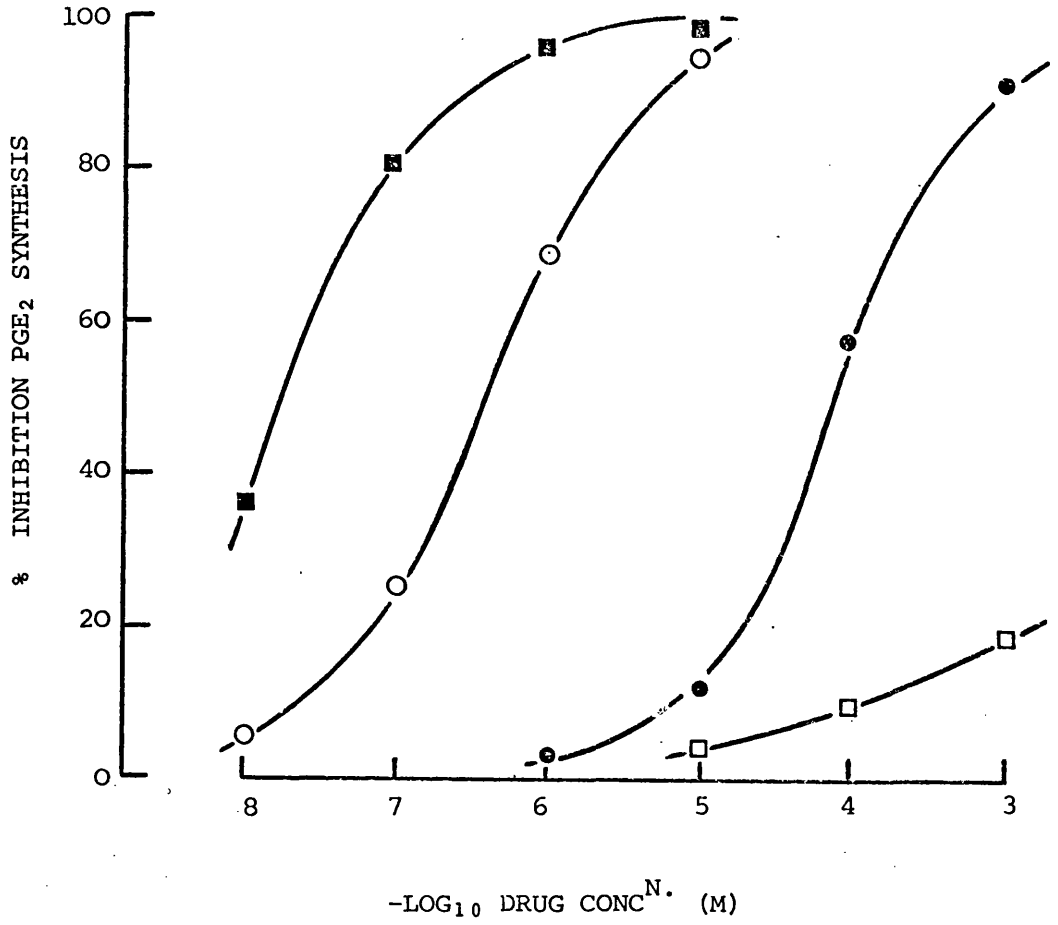
The composite dose-response curve represents the mean of nine consecutive synovial preparations. The mean IC_{50} value was 45μM. Vertical bars indicate the standard error of the mean.

(26μM to 93μM) showed that it was essential to assay aspirin in each synovial preparation. Variations in the IC_{50} values were not due to the different concentrations of enzyme used between experiments as there was no correlation between the microsomal protein concentration used in an experiment and the IC_{50} value obtained from the dose-response curve.

A comparison of the effects of aspirin, paracetamol, indomethacin and flurbiprofen, assayed on the same preparation, is shown in Fig 4.2. Aspirin produced a typical dose-response curve, giving an IC_{50} value of 61μM. However paracetamol (4-acetamidophenol), a drug possessing no anti-inflammatory activity was virtually inactive in this assay, a concentration of 1mM producing only 19% inhibition of PGE_2 production. Other authors have found paracetamol to be inactive against PG synthetase prepared from peripheral tissues such as dog spleen (Flower and Vane, 1972) or rat skin (Greaves and McDonald-Gibson, 1972), though active against the enzyme from dog, rabbit, mouse or gerbil brain (Flower and Vane, 1972; Willis and others, 1972). Indomethacin was found to be considerably more potent than aspirin as an inhibitor of R.A. synovial PG synthetase; its IC_{50} value of 0.24μM meant that on a molar basis it was 257 times more potent than aspirin. Flurbiprofen was found to be a remarkably potent inhibitor (IC_{50} 10.9nM). On a molar basis it was 22 times more potent than indomethacin and no less than 5,600 times more potent than aspirin, the most potent inhibitor of synovial PG synthetase tested.

One drug of particular interest is sodium salicylate which is as effective as aspirin against experimental inflammation, and in the treatment of rheumatoid disease (Collier, 1969), and yet is virtually inactive as an inhibitor of PG synthetase in vitro (Vane,

Fig. 4.2 Inhibition of R.A. synovial PG synthetase by aspirin-like drugs in vitro



Human rheumatoid synovial microsomes (3.1 mg protein/ incubation) were incubated in 0.25M sucrose (0.1M tris-acetate buffer, pH 8.0, with ^{14}C-arachidonic acid (100nCi) for 1 hour at 37°c. Production of ^{14}C-PGE_2 was inhibited by the addition of aspirin (●——●), indomethacin (○——○) and flurbiprofen (■——■). Paracetamol (□——□) was virtually inactive in this respect.

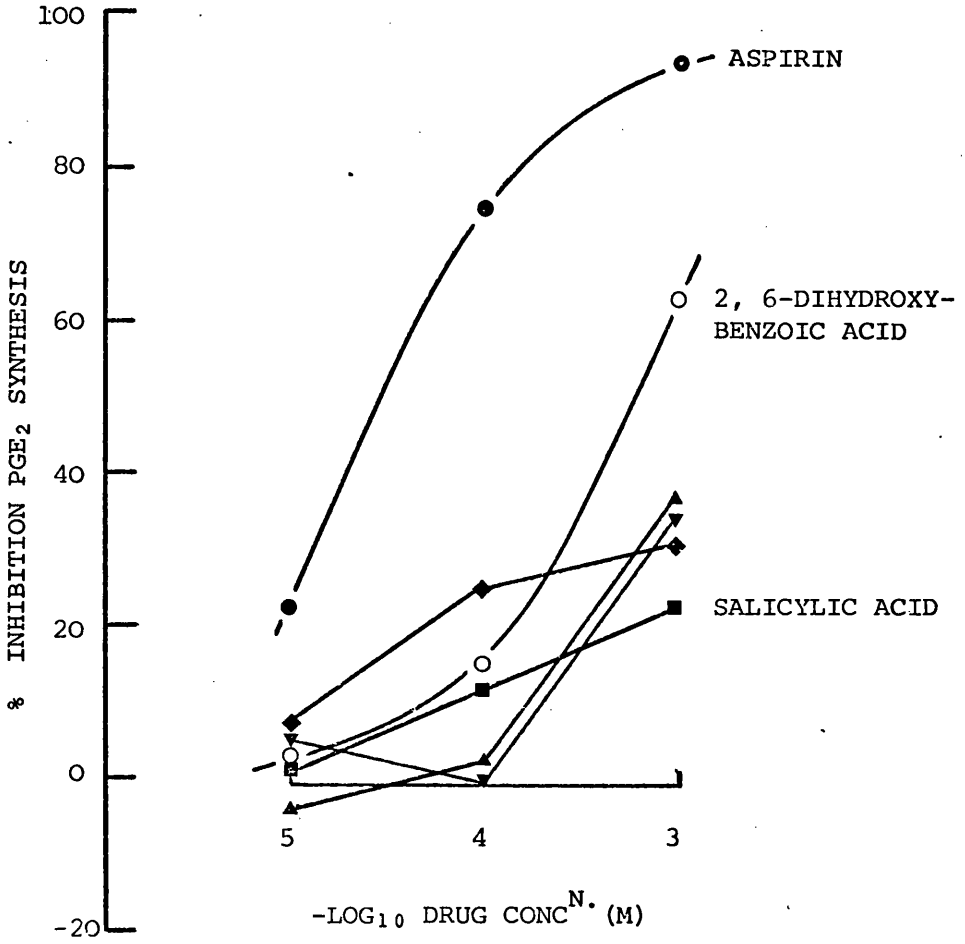
1971; Willis and others, 1972). This apparent discrepancy has led some authors to suggest that salicylic acid requires metabolic transformation before it is fully active in vivo, and that gentisic acid, a metabolite of salicylic acid, possess high antisynthetase activity (Flower and Vane, 1974).

The dose-response curves obtained when aspirin, salicylic acid and various salicylate metabolites were added to R.A. synovial PG synthetase incubations are shown in Fig 4.3. As found with other enzyme preparations salicylic acid was virtually inactive as an inhibitor of PG biosynthesis, producing only 20% inhibition at a concentration of 1mM. (aspirin produced 93% inhibition at this concentration). However, no evidence was found that any of the di-hydroxybenzoic acid compounds studied (including gentisic acid) was a potent inhibitor; even the most potent of the four (2,6-dihydroxybenzoic acid, γ-resorcylic acid) had only $^1/_{20}$ the potency of aspirin as an inhibitor of PG biosynthesis. Other metabolites, such as the acyl or phenolic glucuronides, were not available for testing. The results obtained provide evidence against the possibility that R.A. synovial PG synthetase is sensitive to inhibition by salicylic acid, or its metabolite gentisic acid.

Benorylate, the ester of aspirin and paracetamol, may represent a compound which acquires antisynthetase activity in vivo after metabolic transformation, presumably hydrolysis to aspirin and paracetamol. In vitro, benorylate was inactive as an inhibitor of synovial PG synthetase, though it could not be tested at concentrations greater than 10^{-4}M due to its insolubility.

Table 4.1 shows the relative molar potencies obtained using the present assay for 14 of the commonly used aspirin-like drugs,

Fig. 4.3 Inhibition of R.A. synovial PG synthetase in vitro by aspirin, salicylic acid and salicylate metabolites

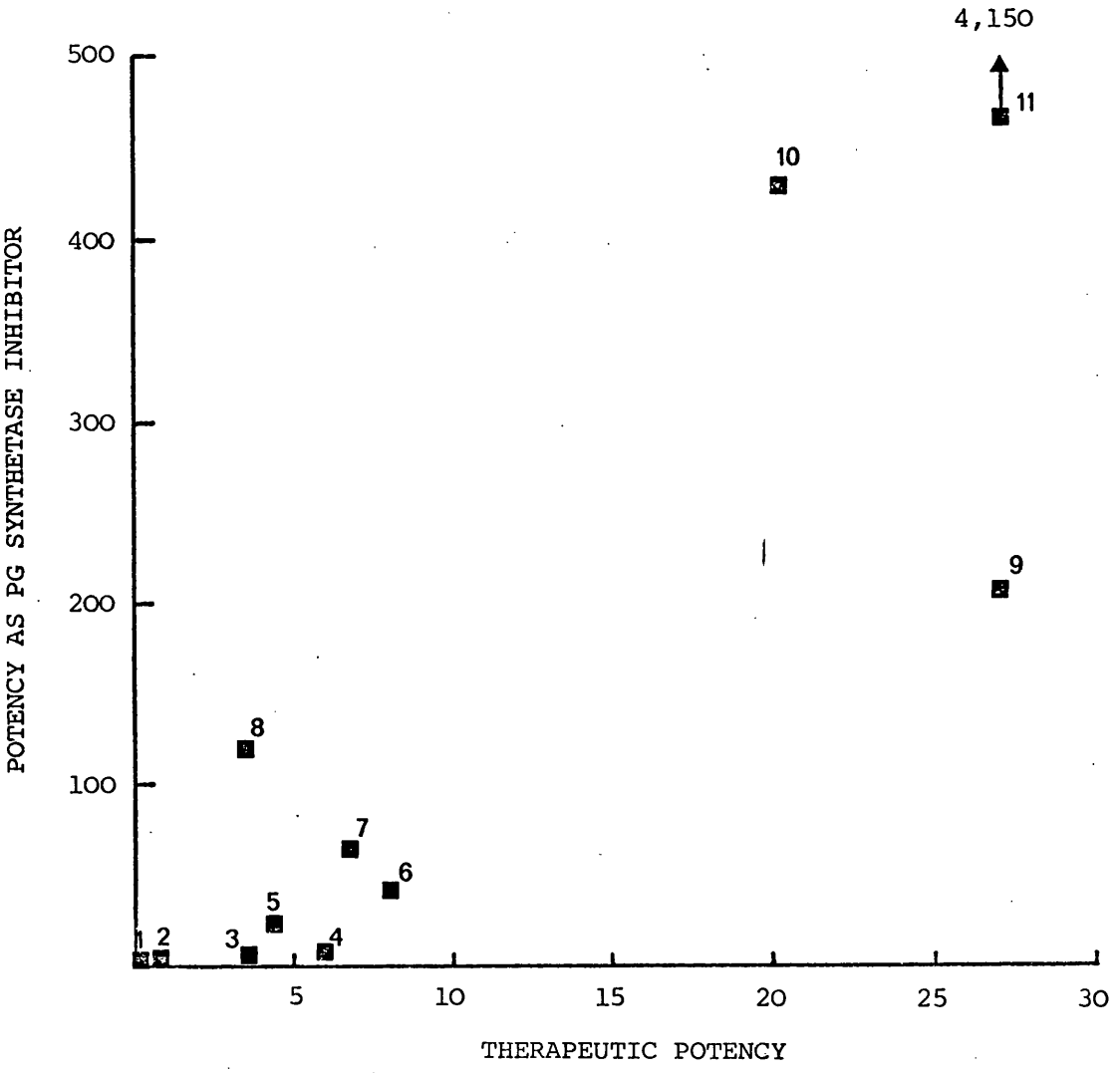


Human rheumatoid synovial microsomes (1.1 mg protein/incubation) was incubated in 0.25M Sucrose/0.1M tris-acetate buffer, pH8.0, with ^{14}C-arachidonic acid (100nCi) for 1 hour at 37^{o}c. Production of ^{14}C-PGE_2 was inhibited by the addition of aspirin (●——●) and, to a less extent, by 2,6-dihydroxybenzoic acid (○——○). 2,3- (◆——◆), 2,4- (▲——▲) and 2,5- (▼——▼) dihydroxybenzoic acid were virtually inactive in this respect, as was salicylic acid (■——■).

Table 4.1 Relative molar potencies of some aspirin-like drugs for 50% inhibition of PGE_2 synthesis

DRUG	RELATIVE MOLAR POTENCY FOR 50% INHIBITION OF PGE_2 SYNTHESIS
PARACETAMOL	< 0.01
SALICYLIC ACID	< 0.02
BENORYLATE	< 0.02
ASPIRIN	1.0
AZOPROPAZONE	2.1
PHENYL BUTAZONE	2.7
FENCLOFENAC	8.1
IBUPROFEN	22
NAPROXEN	45
FLUFENAMIC ACID	110
MEFENAMIC ACID	147
INDOMETHACIN	257
KETOPROFEN	615
FLURBIPROFEN	5,600

Fig. 4.4 A comparison of the potencies of aspirin-like drugs as inhibitors of R.A. synovial PG synthetase in vitro with their therapeutic potencies in clinical practice



1. PARACETAMOL
2. ASPIRIN
3. AZOPROPAZONE
4. PHENYL BUTAZONE
5. IBUPROFEN
6. NAPROXEN
7. FLUFENAMIC ACID
8. MEFENAMIC ACID
9. INDOMETHACIN
10. KETOPROFEN
11. FLURBIPROFEN

Values on both scales are expressed on a weight basis, relative to aspirin.

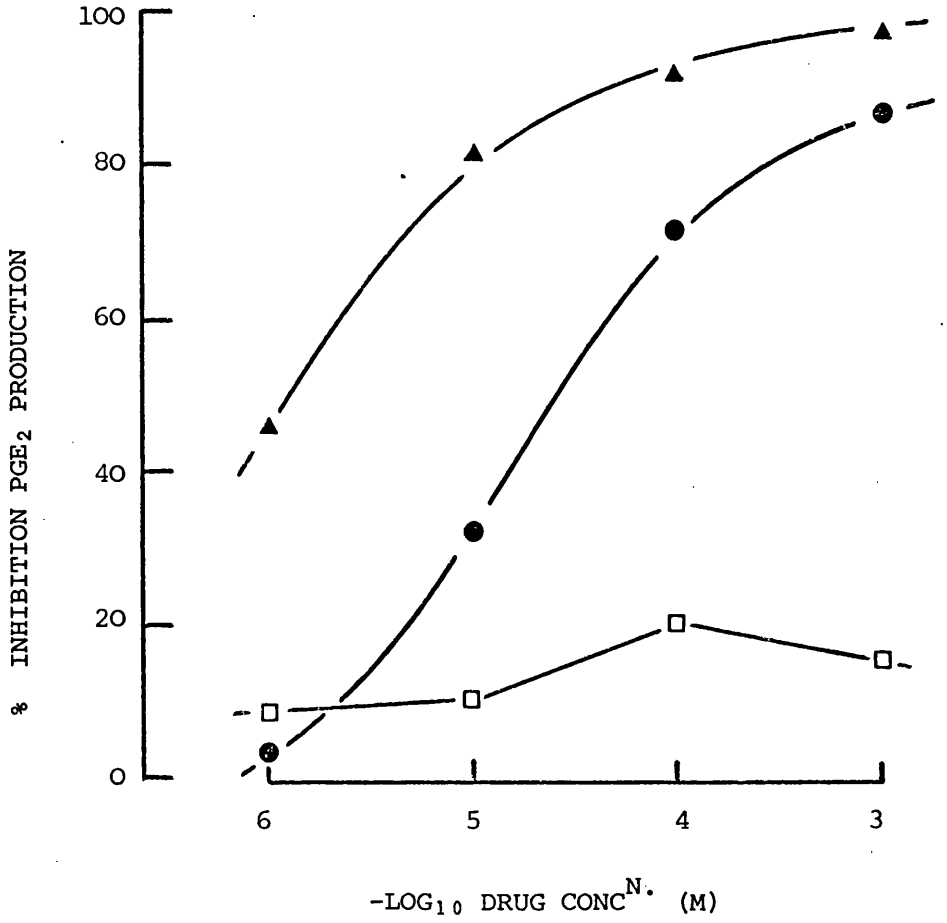
arranged in rank order of potency. From these values Fig. 4.4 was constructed, showing the correlation obtained between the potencies of the aspirin-like drugs as inhibitors of R.A. synovial PG synthetase activity and their known therapeutic potencies in the treatment of rheumatoid disease. Aspirin was taken as unity for each parameter and the relative therapeutic potencies of the drugs assessed from their recommended doses to be found in the 'Monthly Index of Medical Specialities,' and from the typical doses of each drug which clinicians prescribe. Clearly such an assessment of therapeutic potency is inherently imprecise and serves only to illustrate that a relationship does exist between the two parameters. The finding that mefenamic acid is more potent than flufenamic acid as an inhibitor of PG synthetase, but is less potent therapeutically, has been observed by others using the enzyme from sheep seminal vesicles (Ham and others, 1972).

4.3 In vitro inhibition by other antirheumatic drugs

Penicillamine (β'β"-dimethylcysteine) has been increasingly used in the treatment of R.A. since a Multicentre Trial Group (1973) suggested that this drug may actually slow the progression of the disease. Penicillamine cannot be classed as an aspirin-like drug; its slow onset of action and lack of analgesic and antipyretic properties suggest that its mode of action is different from that of the aspirin-like drugs. The finding that this drug is inactive as an inhibitor of R.A. synovial PG synthetase (Fig 4.5) confirms that its anti-inflammatory action is unlikely to be related to inhibition of PG biosynthesis.

The initial reports of Vane (1971) and Ferreira, Moncada and Vane (1971) showed that cortisol was inactive as a PG synthetase inhibitor. Interest was aroused when Greaves and McDonald-Gibson

Fig. 4.5 The effect of penicillamine on R.A. synovial PG synthetase in vitro



Human rheumatoid synovial microsomes (2.4 mg protein/ incubation) were incubated in 0.25M sucrose/0.1 M tris-acetate buffer, pH 8.0, with ^{14}C-arachidonic acid (100nCi) for 1 hour at 37°c. Both aspirin (●——●) and ibuprofen (▲——▲) produced a dose-related inhibition of ^{14}C-PGE_2 synthesis, whereas penicillamine (□——□) was inactive in this respect.

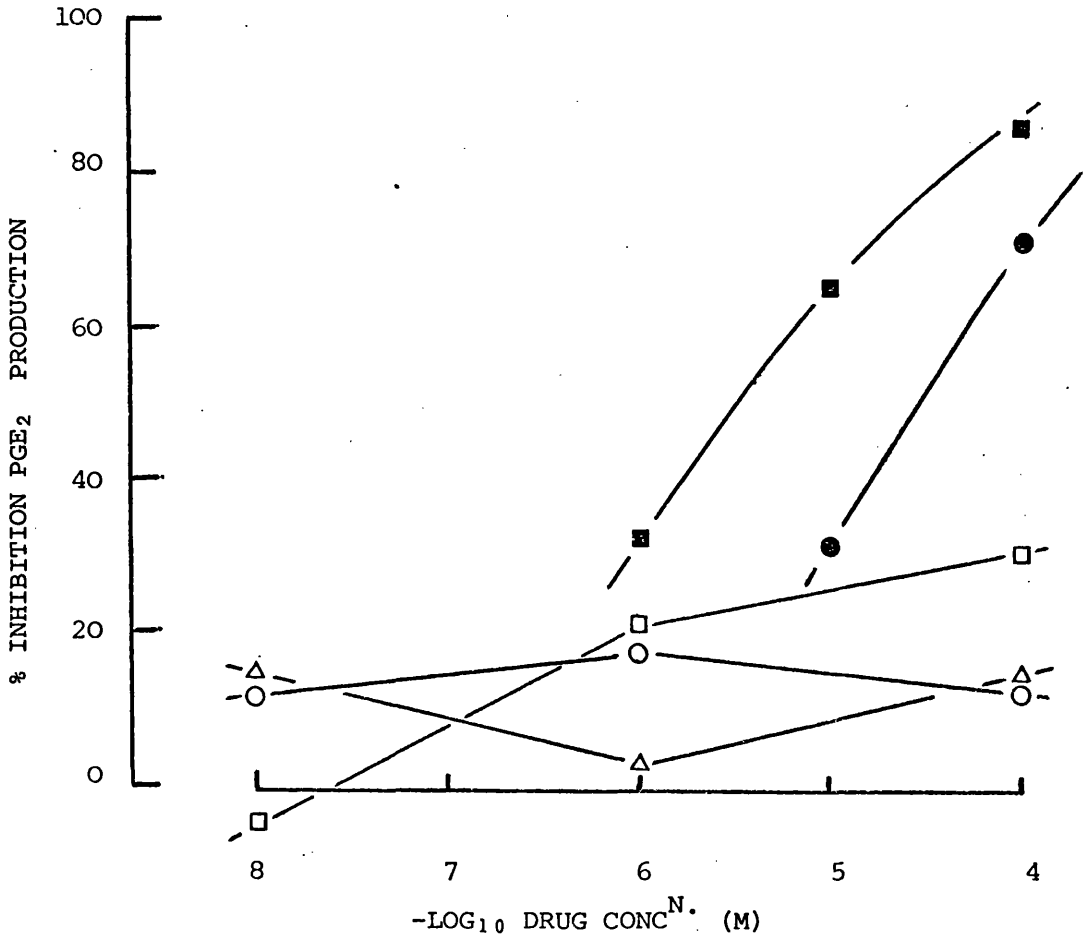
(1972) reported inhibition of PG synthesis in rat skin homogenates by some steroidal anti-inflammatory drugs, though later work using a microsomal preparation of the enzyme showed the steroids to be inactive. Recent work (Hong and Levine, 1976; Nijkamp and others, 1976) suggests that corticosteroids may indeed inhibit production of prostaglandins, but achieve this by blocking release of substrate from cellular phospholipid rather than by directly inhibiting PG synthetase. A study of PG production by rheumatoid synovial cultures (Kantrowitz, Robinson and McGuire, 1975) has shown that cortisol, prednisolone and dexamethazone are able to inhibit PG production in a dose-related manner in the concentration range 10^{-8}M - 10^{-4}M.

Fig 4.6 shows that cortisol, prednisolone and dexamethazone are all ineffective as inhibitors of synovial PG synthesis in vitro. Concentrations greater than 10^{-4}M could not be achieved due to the limited solubility of the drugs, though such concentrations would be far greater than would be achieved therapeutically in vivo; for example, a single dose of prednisolone (15mg) given orally produces peak plasma levels of less than 10^{-6}M (English, Chakraborty and Marks, 1974). The results are in agreement with findings in other tissues that corticosteroids do not inhibit PG synthetase and rule out the possibility that R.A. synovial PG synthetase is uniquely sensitive to inhibition by steroidal anti-inflammatory drugs.

4.4 In vitro inhibition by copper

The presence of copper ions in a homogenate or microsomal preparation of sheep vesicular tissue has been shown to influence PG biosynthesis, producing an increased conversion of substrate to $PGF_{2\alpha}$ and a parallel decrease in PGE_2 synthesis (Lee and Lands,

Fig. 4.6 The effect of anti-inflammatory steroids on R.A. synovial PG synthetase in vitro



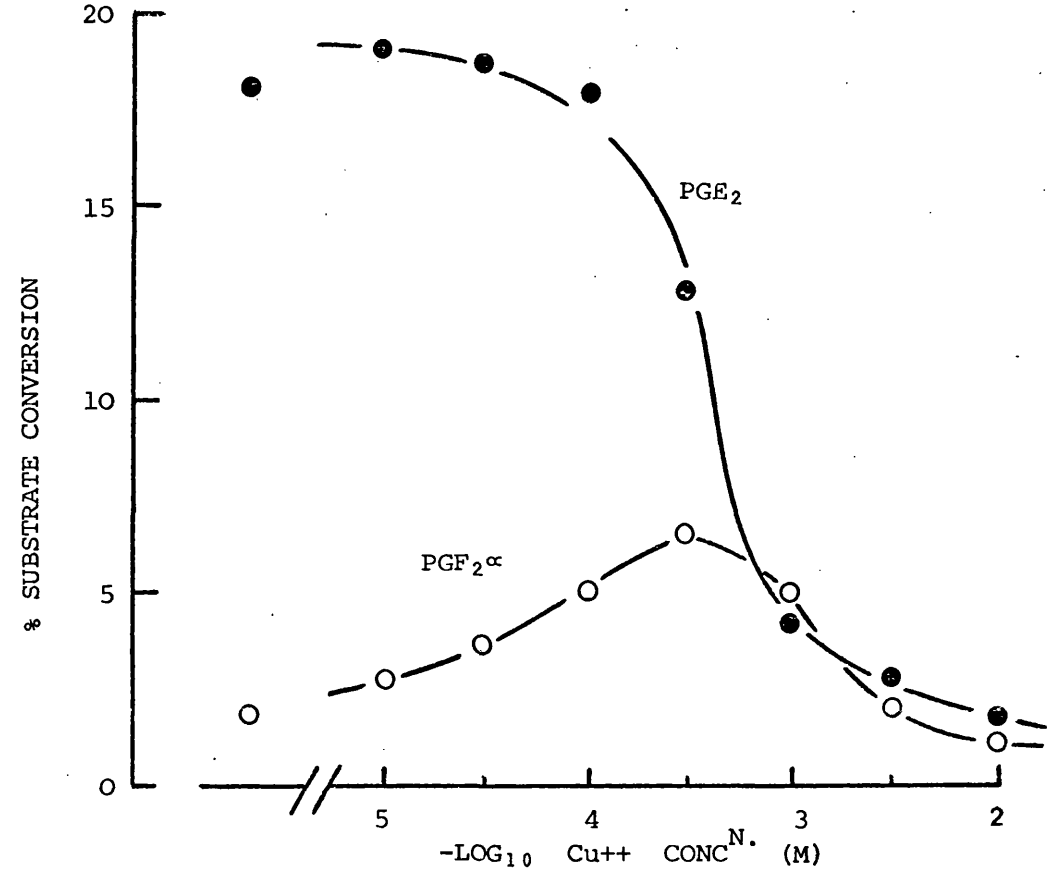
Human rheumatoid synovial microsomes (0.73 mg protein/ incubation) were incubated in 0.25M sucrose/0.IM tris-acetate buffer, pH 8.0, with ^{14}C-arachidonic acid (100nCi) for 1 hour at 37°c. Both aspirin (●——●) and fenclofenac (■——■) produced a dose-related inhibition of ^{14}C-PGE_2 production, whereas cortisol (O——O), prednisolone (Δ——Δ) and dexamethazone (□——□) were virtually inactive in this respect.

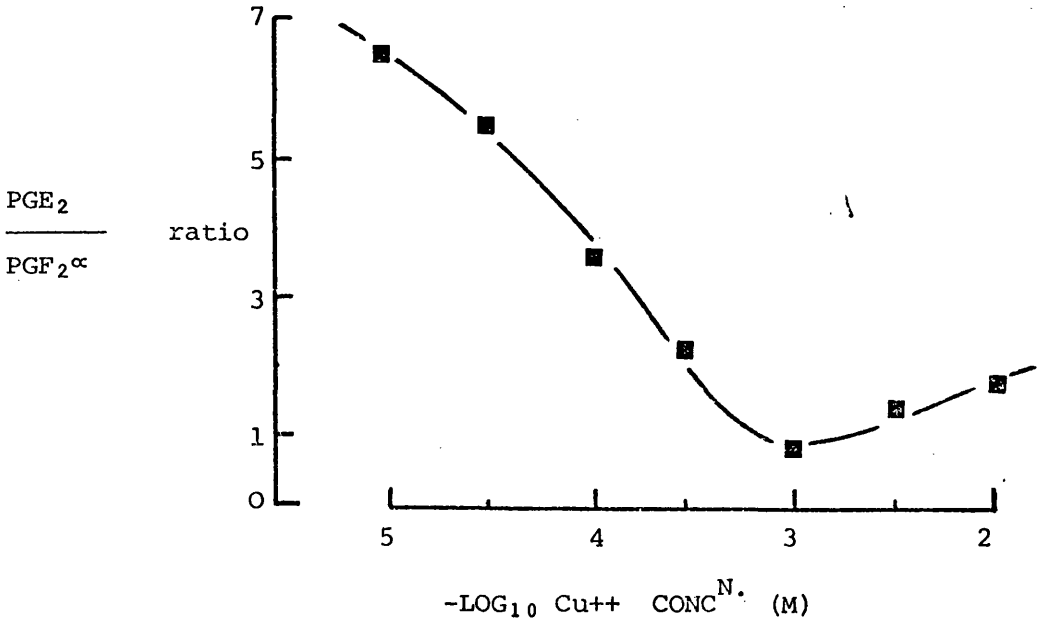
1972; Maddox, 1973). This observation may have therapeutic potential as the E series prostaglandins generally possess the most potent inflammatory properties (section 1.8), the F series being much less potent and in some cases antagonising the actions of the E compounds. The finding that copper acetate has an anti-inflammatory action in an experimental model of inflammation (Sorenson, 1976) provides some evidence that such a mechanism of action may exist in vivo and is not merely an in vitro phenomenon. This author has suggested that copper chelates may be the 'active' forms of several antiarthritic agents and has shown that these complexes are more potent anti-inflammatory agents than their parent compounds. At the same time they are not only less ulcerogenic than the parent compounds but actually possess anti-ulcer activity, clearly an extremely attractive therapeutic goal. A study of the effects of copper aspirin and copper salicylate by Rainsford and Whitehouse (1976) however was less optimistic and suggests that further work is warranted.

The results of adding copper ions (as cupric acetate) to R.A. synovial microsomes, incubated with ^{14}C-arachidonic acid, are shown in Fig. 4.7. As can be seen copper produces a dose-related inhibition of PGE_2 synthesis, giving an IC_{50} value of 4×10^{-4}M. However the effect on $PGF_{2\alpha}$ production is markedly different; a stimulation of $PGF_{2\alpha}$ synthesis is achieved over the range 10^{-5} - 10^{-3}M and inhibition is only seen at high concentrations (above 3×10^{-3}M). A plot of the ratio of PGE_2 to $PGF_{2\alpha}$ produced shows that this value is a minimum (0.8) at a copper concentration of 10^{-3}M, though maximum stimulation of $PGE_{2\alpha}$ synthesis is achieved at 3×10^{-4}M.

The effect of adding copper aspirin to synovial incubates is shown in Fig. 4.8, together with the dose-response curves obtained

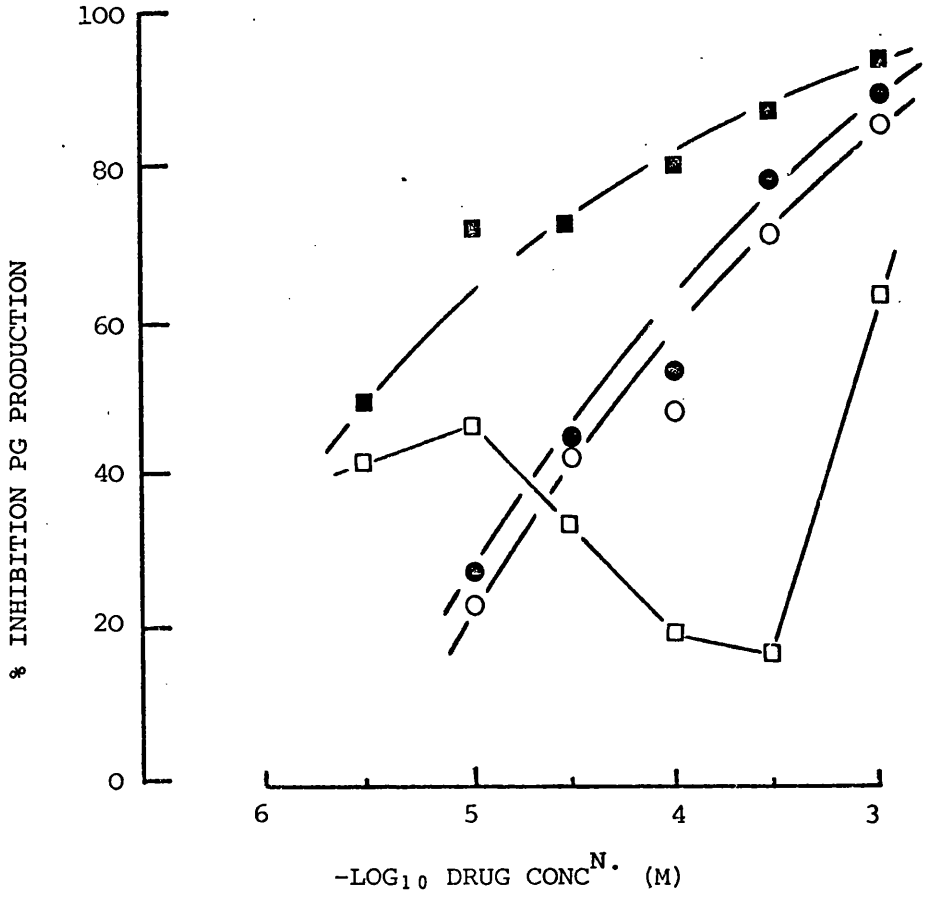
Fig. 4.7 The effect of copper on R.A. synovial PG synthetase in vitro





Incubations were carried out as described previously, except that EDTA was omitted from the buffer.

Fig. 4.8 The effect of copper aspirin on R.A. synovial PG synthetase in vitro



Human rheumatoid synovial microsomes (0.65 mg protein/ incubation were incubated in 0.25M sucrose/0.1M tris-acetate buffer (without EDTA), pH 8.0, with ^{14}C-arachidonic acid (100nCi) for 1 hour at 37°c. Production of PGE_2 (closed symbols) and $PGF_{2\alpha}$ (open symbols) was inhibited by the addition of aspirin (●—●, ○—○) and copper aspirin (■—■, □—□).

by adding aspirin. Aspirin produced the expected dose-related inhibition of both PGE_2 and $PGF_{2\alpha}$ synthesis, giving an IC_{50} value calculated from either product of approximately 60μM. The effect of copper aspirin was quite different; while PGE_2 production was inhibited in a dose-related manner, but to a greater extent than with aspirin (IC_{50} 3μM), inhibition of $PGF_{2\alpha}$ synthesis was not dose-related, being less than that produced by aspirin (at least over the range $3 \times 10^{-5}M - 10^{-3}M$) and having a minimum at $3 \times 10^{-4}M$. No stimulation of $PGF_{2\alpha}$ synthesis was observed, however, as was the case with copper ions alone (Fig. 4.7).

4.5 In vivo inhibition of PG synthetase

The results presented in this chapter were obtained using 31 synovial tissue samples taken from 27 patients (23 female, 4 male) who had undergone surgical synovectomy of the knee or, in one case (patient 17), the elbow. All had classical R.A. with a positive latex test. Brief clinical details of these patients are shown in Table 4.2, together with the weight of each tissue, trimmed of fat, and a measure of the vascularity and degree of fibrosis, both scored on an arbitrary scale of 0 - +++.

It was of particular interest to study the influence of aspirin-like drug therapy on subsequent PG synthetase activity *in vitro*, as both aspirin and indomethacin, for example, have been reported to be irreversible inhibitors of PG synthetase in other systems (Smith and Lands, 1971; Raz, Stern and Kenig-Wakshal, 1973).

Fig. 4.9 shows the amounts of PGE_2 and $PGF_{2\alpha}$ produced by the microsomal preparations from each of the 31 synovia studied. Tissues from seven patients receiving indomethacin therapy (patients 1-7) were all capable of PG synthesis *in vitro* (mean amount of PGE_2 produced

Table 4.2

Patient No.	Sex	Age	Duration of R.A. (yrs)	Aspirin-like drugs	Weight of tissue (g)	Vascularity	Fibrosis	PG produced pmol/mg/hr E_2	$F_{2\alpha}$
1	F	45	8	Indomethacin 100 mg/day	14	+++	+	198	13
2	F	22	8	Indomethacin 75 mg/day	17	+++	+	66	7
3	F	65	12	Indomethacin 100 mg/day	8	0	++	292	18
4	M	63	24	Indomethacin 75 mg/day	63	+	++	123	6
5(a)	F	51	10	Indomethacin 75 mg/day	12	0	0-+	395	68
6	F	37	7	Indomethacin 75 mg/day	48	+++	0-+	120	21
7	F	55	10	Indomethacin 75 mg/day	26	+++	0	76	7
8	F	67	17	Naproxen 500 mg/day	21	++	0	32	4
9	F	52	14	Naproxen 750 mg/day	13	+	0-+	493	47
10	F	63	4	Ibuprofen 1200 mg/day	42	++	+	133	14
11(a)	F	38	5	Ibuprofen 1600 mg/day	29	+	+	81	15
11(b)	F	38	5	Ibuprofen 1600 mg/day	54	+	+	69	23

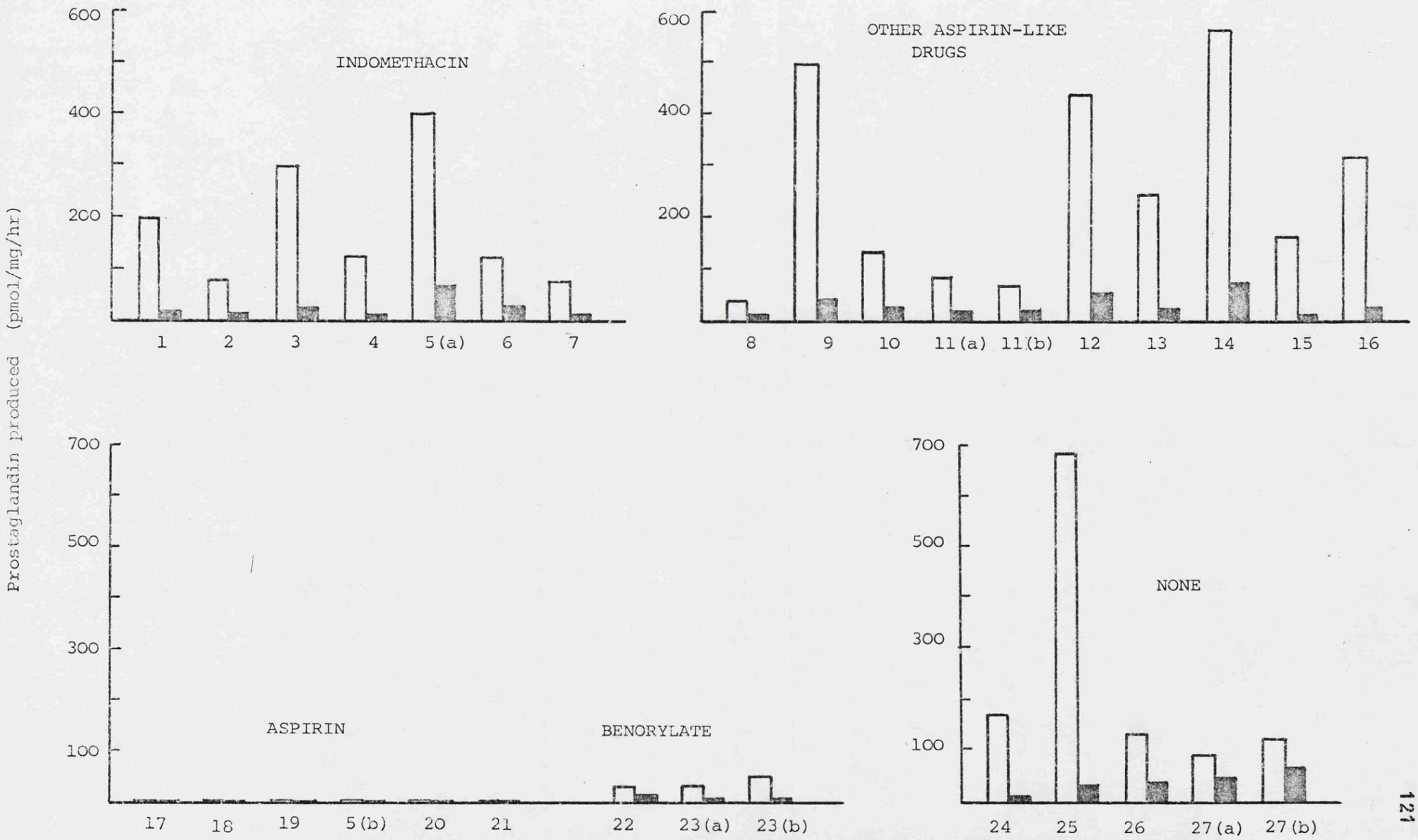
Table 4.2 (continued)

Patient No.	Sex	Age	Duration of R.A. (yrs)	Aspirin-like drugs	Weight of tissue (g)	Vascularity	Fibrosis	PG produced pmol/mg/hr E_2	$F_{2\alpha}$
12	F	32	7	Ibuprofen 800 mg/day	10	O-+	++	442	56
13	F	62	5	Fenoprofen 1800 mg/day	51	+	O	248	24
14	F	33	6	Fenclofenac 600 mg/day	23	++	+	569	73
15	M	57	12	Azopropazone 900 mg/day	30	O-+	++	168	13
16	F	55	22	Phenyl butazone 100 mg/day	25	O-+	O-+	325	29
17	F	60	23	Aspirin 3.6 g/day	18	++	O	2	<1
18	F	23	2	Aspirin 2.4 g/day	20	+	+	9	1
19	F	68	8	Aspirin 1.2 g/day	31	+	+	7	<1
5(b)	F	51	10	Aspirin 600 mg/day	9	+	++	8	1
20	F	67	14	Aspirin 600 mg/day	3	O-+	O-+	8	<1
21	F	60	11	Aspirin 600 mg/day	25	++	+	2	<1

Table 4.2 (continued)

Patient No.	Sex	Age	Duration of R.A. (yrs)	Aspirin-like drugs	Weight of tissue (g)	Vascularity	Fibrosis	PG produced pmol/mg/hr E_2	$F_{2\alpha}$
22	F	54	3	Benorylate 8.0 g/day	24	+	+	35	18
23(a)	M	60	8	Benorylate 8.0 g/day	19	+	+	35	8
23(b)	M	60	8	Benorylate 8.0 g/day	29	+	+	53	9
24	F	57	18	None	30	+++	0	173	12
25	M	49	15	None	27	++	+	687	28
26	F	66	12	None	6	0	0	126	42
27(a)	F	52	30	None	14	+	+	88	48
27(b)	F	52	30	None	10	+	+	118	63

Fig. 4.9 The effect of aspirin-like drug therapy on subsequent R.A. synovial PG synthetase activity in vitro



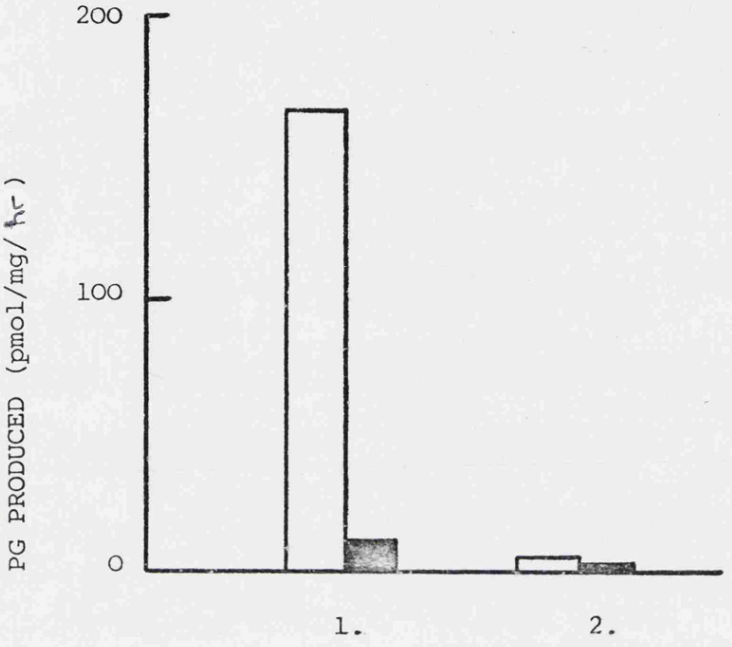
Numbers refer to the patients in Table 4.2.

171 pmol/mg/hr, range 66-395 pmol/mg/hr). Similarly ten samples from patients receiving other aspirin-like drugs (patients 8-16) gave a mean PGE_2 production of 256 pmol/mg/hr, range 32-569 pmol/mg/hr. However, in six tissues from patients receiving aspirin, (patients 5(b) and 17-21) the amounts of PGE_2 produced were virtually indistinguishable from zero (mean 6 pmol/mg/hr, range 2-9 pmol/mg/hr). Five samples from patients receiving no aspirin-like drugs (patients 24-27) produced about the same levels of PGE_2 as did the groups being maintained on indomethacin and other aspirin-like drugs (mean value 238 pmol/mg/hr, range 88-687 pmol/mg/hr). Three preparations from two patients taking benorylate (patients 22 and 23) produced small but measurable amounts of PGE_2 (mean 41 pmol/mg/hr, range 35-53 pmol/mg/hr).

These findings may be explained if the inhibition of R.A. synovial PG synthetase by aspirin *in vivo* is considered to be of a non-competitive (irreversible) type, while the *in vivo* inhibition caused by indomethacin and the other aspirin-like drugs studied here is considered to be competitive (reversible). In this latter case the manipulation of the tissue through the preparative stages described would be expected to remove the drug from the enzyme receptor site, reducing its concentration to a very low level and permitting virtually uninhibited enzyme activity *in vitro*. It may be that such a kinetic situation exists *in vivo* when the drugs are given therapeutically.

An opportunity arose to study the synovia obtained from the opposite knees of one patient, (patient 5) the first during indomethacin therapy and the second, two months later while the patient was receiving aspirin. As shown in Fig. 4.10 the amount of enzyme activity when the patient was receiving aspirin, even at the low dose of 600 mg/day, was only 2% of that found in the opposite knee some 2 months previously when the patient was taking indomethacin

Fig. 4.10 PG synthetase activity of R.A. synovial microsomes in vitro from opposite knees after indomethacin or aspirin therapy



The production of PGE_2 □ and $PGF_{2\alpha}$ ■ was measured in vitro using the standard technique described in sections 2.3 - 2.5.

1. Patient taking indomethacin, 75 mg/day.
2. Opposite knee of same patient two weeks later, indomethacin therapy replaced with aspirin, 600 mg/day.

(75 mg/day). This marked different between the two synovia is most unlikely to be due solely to the difference in activity between one synovia and the next, for in three other patients (patients 11, 23 and 27) who underwent simultaneous bilateral synovectomies the PG synthetase activities found in opposite knees were in reasonably good agreement (Fig 4.9).

No correlation was observed between the level of PG synthetase activity measured in the microsomal fraction of each tissue and other parameters such as the length of the patient's history, the weight of the tissue or its degree of vascularity or fibrosis.

4.6 Discussion

The aspirin-like drugs have been shown to be potent inhibitors of PG biosynthesis, both *in vitro* and *in vivo*, in a wide range of human and animal tissues (Flower, 1974). It was not surprising therefore that PG synthetase prepared from R.A. synovial tissue was found to be susceptible to inhibition by low concentrations of the aspirin-like drugs. However if the interaction between PG synthetase and the aspirin-like drugs *in vitro* is to be relevant to proposed *in vivo* effects, then it must be elicited at drug concentrations equivalent to those occurring in man during aspirin-like drug therapy.

Taking aspirin as an example, the maximum plasma salicylate concentration of a rheumatoid patient receiving 6g/day of aspirin in divided doses is of the order of 1-2mM, the concentration of free salicylate, i.e. that fraction of the drug not bound to either circulating or tissue proteins, being 0.1-0.6mM. (Smith and Dawkins, 1971). In the present study the IC_{50} values for aspirin, ranging from 26 to 93μM, thus fall well within the range of concentration attainable in man during therapy. Similarly, the maximum plasma concentrations of

indomethacin achieved in man are approximately 5μM 'total' or 0.5μM 'free' (Hucker and others, 1966) whereas the IC_{50} values measured for indomethacin in the present work were in the range 0.1-0.37μM.

The IC_{50} values found in the present work are in marked contrast to some other values reported in the literature; using a bovine seminal vesicle preparation, Flower, Cheung and Cushman (1973) reported an IC_{50} value for aspirin of 9mM for PGE_2 formation, and >10mM for the formation of $PGF_2\alpha$, PGD_2 and malondialdehyde. These concentrations would not be achieved in man even during acute aspirin poisoning , and in the present author's opinion are a reflection of the unrealistic substrate concentration employed (1mM). Similarly the reported IC_{50} values for indomethacin of 20-40μM are far above the concentration attainable in man during normal therapy. Tomlinson and others (1972), using a substrate concentration of 0.33mM with bovine seminal vesicles, reported an IC_{50} value for aspirin of 15mM and Smith and Lands (1971), using 60μM substrate with sheep seminal vesicles, found an IC_{50} of 9mM for aspirin. That the enzyme from bovine seminal vesicular glands is not peculiarly insensitive to inhibition by aspirin or indomethacin has been shown by Cushman and Cheung (1976); using a substrate concentration of 1μM with bovine seminal vesicles these authors reported the much more realistic values of 400μM and 0.6μM for the IC_{50} of aspirin and indomethacin respectively. Such findings would appear to justify the low substrate concentration (1μM) used in the present studies.

The finding that paracetamol is virtually inactive as an inhibitor of R.A. synovial PG synthetase is in agreement with findings in other peripheral tissues (Flower and Vane, 1972; Greaves and McDonald-Gibson, 1972). As paracetamol possesses no anti-inflammatory activity this result is in accord with Vane's hypothesis.

As discussed previously the susceptibility of brain PG synthetase to inhibition by paracetamol may explain the drug's anti-pyretic activity, as it is well established that paracetamol is a centrally-acting anti-pyretic. The analgesic action of paracetamol is less readily explained on this basis, however, as it is generally agreed that the aspirin-like drugs are peripherally-acting analgesics (Guzman, Braun and Lim, 1962; Lim and others, 1964), in contrast to the centrally-acting narcotic analgesics. As discussed shortly, it may be an indication that the aspirin-like drugs are able to exert their effects by mechanisms other than their well-documented inhibition of PG biosynthesis.

Salicylic acid was found to be virtually inactive as an inhibitor of R.A. synovial PG synthetase in vitro, in agreement with the findings in other systems (Vane, 1971; Willis and others, 1972). These results present a difficulty for the Vane hypothesis, for aspirin and sodium salicylate are known to be equally effective in the treatment of R.A. (Woodbury, 1965), and equipotent in a number of experimental models of inflammation in animals (Collier, 1969; Willis and others, 1972; Smith, Ford-Hutchinson and Elliott, 1975).

Several suggestions have been advanced to explain this discrepancy. One is that different tissues contain isoenzymes of PG synthetase, each possessing a characteristic pharmacological 'profile' in respect of inhibition by various aspirin-like drugs (Vane, 1971); salicylic acid could thus be a potent inhibitor of PG synthesis in inflammatory tissue yet be ineffective as an inhibitor in other tissues. The results obtained in the present work provide evidence against the possibility that human inflammatory tissue contains an isoenzyme of PG synthetase which is susceptible to inhibition by salicylic acid.

It has also been proposed (Vane, 1972; Willis and others, 1972) that salicylic acid requires metabolic transformation before it acquires anti-synthetase activity *in vivo*, and that gentisic acid, a metabolite of salicylic acid, possesses high anti-synthetase activity (Flower and Vane, 1974). The results obtained in the present study do not support this explanation; gentisic acid was virtually inactive as an inhibitor of synovial PG synthetase and even the most potent of the metabolites tested (γ-resorcylic acid) was only $\frac{1}{20}$ as potent as aspirin. On purely hypothetical grounds this explanation would seem somewhat unlikely. Firstly, gentisic acid and other dihydroxybenzoic acids represent only 1-5% of the metabolites of salicylic acid (Alpen and others, 1951; Kapp and Coburn, 1942). As gentisic acid was reported to be only 1.5 times more potent than aspirin as an inhibitor of PG synthetase (Flower and Vane, 1974) this could hardly account for the equipotency of aspirin and salicylic acid as anti-inflammatory agents.

Secondly, gentisic acid itself possesses no anti-inflammatory activity in various experimental models of inflammation (Adams and Cobb, 1967) and is useless as a therapeutic agent in the treatment of rheumatoid disease (Bywaters, 1963). Finally, it is well known that *in vivo* aspirin is rapidly hydrolysed to salicylic acid and subsequently gives rise to the same pattern of metabolites as salicylic acid itself (Levy and Leonards, 1966). By implication aspirin would also give rise to any salicylic acid metabolite possessing anti-synthetase activity, making it difficult to envisage how salicylic acid could achieve the same therapeutic potency as aspirin.

Smith, Ford-Hutchinson and Elliott (1975) have presented evidence that an important component of the experimental anti-inflammatory action of sodium salicylate and aspirin involves interference with cellular infiltration. This action would not

involve a primary effect on PG synthesis, in which case the *in vivo* effects of sodium salicylate on the PG content of inflammatory exudates (Willis and others, 1972) would be secondary to an initial effect on leucocyte migration. This secondary inhibition of PG production is quite possible, as leucocytes are known to release large amounts of phospholipase A and prostaglandins during phagocytosis (Higgs and Youlten, 1972).

A plot of the relative potencies of the aspirin-like drugs as inhibitors of R.A. synovial PG synthetase against their known therapeutic potencies in the treatment of rheumatoid disease showed that a relationship exists between the two parameters, albeit a somewhat imprecise one. As a drug has to overcome problems of absorption, distribution and metabolism before reaching its 'target' *in vivo* it is perhaps not to be expected that the correlation between an *in vitro* and *in vivo* property of a drug would be an exact one. As exemplified by flurbiprofen, the *in vitro* assay may, with some drugs, give a grossly exaggerated indication of their *in vivo* potency. Clearly an *in vitro* assay is always subject to such limitations, particularly so in the case of PG synthetase as so little is known of the factors controlling the activity of the enzyme in its intracellular environment *in vivo*. Furthermore, as discussed above, it may be an oversight to consider inhibition of PG biosynthesis as the sole mode of action of the aspirin-like drugs without taking into account their actions on other aspects of the inflammatory process.

The finding that cortisol, prednisolone and dexamethazone are inactive as inhibitors of R.A. synovial PG synthetase over the concentration range 10^{-8}-10^{-4}M is in agreement with the original reports of Vane (1971) and Ferreira, Moncada and Vane (1971) that

corticosteroids do not inhibit PG synthetase. A recent finding of great interest (Nijkamp and others, 1976) is that corticosteroids inhibit the activity of rabbit aorta contracting substance-releasing factor (RCS-RF) and that the relative activities of nine representative corticosteroids in blocking this RCS-RF activity closely parallel their known anti-inflammatory potencies. As it is thought that RCS-RF may act via phospholipase A_2 to release free arachidonic acid from cellular phospholipids and hence bring about increased synthesis of prostaglandin endoperoxides and thromboxanes, it is possible that corticosteroids may exert their anti-inflammatory actions, in part at least, through this indirect inhibition of PG biosynthesis.

The influence of copper on PG biosynthesis is of great interest, being able to bring about an inhibition of PGE_2 synthesis and a simultaneous increase in $PGF_{2\alpha}$ production. As discussed previously this may have therapeutic importance as the E type prostaglandins usually are the most potent in exerting inflammatory properties. This 'selective' PGE inhibition is not usually seen with the aspirin-like drugs, though phenylbutazone has been reported to be a selective PGE_2 inhibitor in one _in vitro_ system (Stone, Mather and Gibson, 1975). A claim that phenylbutazone inhibited production of PGE_2 and $PGF_{2\alpha}$ but not PGD_2 (Flower, Cheung and Cushman, 1973) was later retracted (Cushman and Cheung, 1976).

In the present studies the concentrations of copper necessary to produce these effects were higher than would be found _in vivo_; the IC_{50} for inhibition of PGE_2 synthesis was 400μM, whereas in plasma the mean total copper concentrations in normals and R.A. patients are approximately 25 and 35μM respectively (Niedermeier, 1965). The corresponding concentrations of non-ceruloplasmin copper are 4μM and

6μM respectively. Values for non-ceruloplasmin copper in synovial fluid are 6μM for normal volunteers and 4μM for R.A. patients. The high concentrations needed to elicit the effects shown in the present work may be due to the incubation conditions used, as GSH has been shown to partially reverse the inhibitory effects of Zn^{2+}, Cd^{2+} and Cu^{2+} ions (Nugteren, Beerthuis and van Dorp, 1966). The results emphasise the need for caution when extending *in vitro* findings to speculation of a possible action *in vivo*.

The finding that copper aspirin was able to bring about a greater degree of inhibition of PGE_2 synthesis than aspirin itself, together with a markedly reduced inhibition of $PGF_{2\alpha}$ synthesis is of interest as Sorenson (1976) has shown that copper coordination complexes of the aspirin-like drugs possess greater anti-inflammatory activity in animal models than the parent compounds, together with much lower toxicity. It has been suggested that copper complexes are possibly the 'active' forms of the aspirin-like drugs *in vivo*, and that unwanted side effects such as gastric irritation may be due to depletion of copper in tissues such as the gastric mucosa which may require copper for their normal maintenance and function (Elmes, 1974).

It has also been suggested that copper complexes may protect synovial fluid from degradation, and leucocytes from premature death, by dismutation of superoxide anion, thereby preventing the formation of potentially damaging hydroxyl radicals (Richardson, 1976). The test of all the above hypotheses will be whether such *in vitro* effects can be observed *in vivo* and ultimately whether copper complexes are in fact potent, relatively non-toxic drugs in the treatment of rheumatoid disease in man.

A recent study (Boyle and others, 1976) has examined the ability of several aspirin-like drugs to cause ulceration when administered

orally in the rat. The damage caused by clopirac, niflumic acid and aspirin was virtually abolished when they were given as copper complexes whereas the damage caused by indomethacin, ketoprofen and aspirin was unaltered. The lack of ulceration of the first three copper complexes was associated with a much reduced ability to inhibit PG synthesis in an in vitro system and suggests that inhibition of PG synthesis in vivo may be more relevant to ulcerogenicity than anti-inflammatory activity. All six copper complexes were found to be equipotent with their parent compounds in the carrageenan-induced oedema test, in contrast to the report of Sorenson (1976) that the copper complexes possessed greater anti-inflammatory activity. The difference may be due to the route of administration of the drug, for the former authors gave the drugs orally whereas the latter used subcutaneous administration.

It was of interest to find that microsomal preparations of synovial tissue from patients receiving indomethacin and other aspirin-like drugs were capable of considerable PG synthesis in vivo as both aspirin and indomethacin have been reported to be irreversible inhibitors of the enzyme prepared from sheep seminal vesicles (Smith and Lands, 1971; Ku and Wasvary, 1973; Raz, Stern and Kenig-Wakshal, 1973). In contrast, there appeared to be a fundamental difference in this respect after aspirin therapy; in vitro PG synthetase activity was virtually undetectable in the preaprations from six patients who were receiving aspirin therapy at the time of synovectomy. These findings suggest that aspirin may be unique in being an irreversible inhibitor of R.A. synovial PG synthetase whereas other aspirin-like drugs such as indomethacin, naproxen and ibuprofen are reversible inhibitors.

Such a difference in the modes of action of aspirin and indomethacin has not been reported in any other system, though the report of Raz, Stern and Kenig-Wakshal (1973) is interesting in the light of

the present results. These authors found that both aspirin and indomethacin were irreversible inhibitors of sheep seminal vesicle PG synthetase (using an acetone-pentane powder preparation). However, when fresh slices of seminal vesicular tissue were pre-incubated with either aspirin or indomethacin, enzyme activity in microsomes prepared from the aspirin-treated slices was markedly inhibited while the activity in microsomes from indomethacin-treated slices was unaffected. The authors concluded that indomethacin may fail to reach the enzyme in an intact cell and questioned whether inhibition of PG synthesis by indomethacin *in vivo* is mediated via direct inhibition of PG synthetase. To the present author a more likely explanation is that indomethacin is a reversible inhibitor of PG synthetase in fresh tissue or microsomes, but an irreversible inhibitor when the enzyme is prepared as an acetone-pentane powder and is denuded of its normal lipid environment. Aspirin would appear to be an irreversible inhibitor of either preparation.

The above explanation is supported by the findings that indomethacin is a competitive inhibitor of fresh microsomal preparations of sheep and bovine seminal vesicle PG synthetase (Hamberg, 1972), but an irreversible inhibitor on an acetone-dried powder preparation (Smith and Lands, 1971). These findings, together with the results presented in the present work suggest that if this phenomenon is a general one, then acetone-pentane powder preparations of PG synthetase may produce *in vitro* results which are not relevant to the activity of the enzyme *in vivo* in its natural micro-environment.

A further finding of interest was that PG synthetase activity was abolished in microsomal preparations from three patients receiving only 600 mg/day of aspirin. Although such a dose is capable of exerting an analgesic effect in man it is well known that much larger doses are necessary to produce a clinical antirheumatic effect (Boardman and

Hart, 1967). If a small dose of aspirin is able to abolish PG synthetase activity in the target tissue, and if one accepts the hypothesis that aspirin exerts its anti-inflammatory effects via inhibition of PG synthesis, then it is pertinent to ask why much larger doses are necessary in the treatment of rheumatoid disease. In the present author's opinion these findings support the view of Smith, Ford-Hutchinson and Elliott (1975) that the anti-inflammatory efficacy of aspirin may have important components other than its well-documented inhibition of PG synthesis.

As discussed by Whitehouse (1974) the aspirin-like drugs are able to modulate several molecular or cellular events concerned in the initiation and maintenance of inflammatory responses. It may be an oversight therefore to consider the anti-inflammatory and antirheumatic actions of these drugs solely in terms of inhibition of PG synthesis. Further elucidation of other facets of their mode of action may well be assisted by the design of new anti-inflammatory agents which are not inhibitors of PG synthetase(s). The use of such compounds might also indicate to what extent the undesirable side-effects of the aspirin-like drugs are due to inhibition of PG synthesis in tissues other than the target tissue. The present author suspects that the design and synthesis of a new drug possessing a high degree of specificity for the PG synthetase of human inflammatory target tissue is inherently unlikely.

CHAPTER FIVE

STUDIES WITH HUMAN LEUCOCYTE PG SYNTHETASE

5.1 Introduction

The results obtained in the previous two chapters demonstrate that a microsomal fraction prepared from human rheumatoid synovial tissue is capable of considerable PG biosynthesis *in vitro*, and that this synthesis may be abolished by low concentrations of the aspirin-like drugs, i.e. concentrations likely to be achieved in the blood during anti-inflammatory therapy.

As emphasised by Willoughby and others (1973) a knowledge of the source of the prostaglandins found at an inflammatory site is essential to an understanding of the mode of action of the aspirin-like drugs, though differing views exist on this point. The original papers of Vane (1971), Smith and Willis (1971) and Ferreira, Moncada and Vane (1971) initiated the hypothesis that prostaglandins are formed at the site of inflammation, and that the aspirin-like drugs act at the site to abolish PG formation. Furthermore, Piper and Vane (1971) suggested that tissues do not store prostaglandins, and that PG release could thus be equated with synthesis. This is in accord with the report of Jouvenaz and others (1970) that in tissues extracted in such a way as to prevent PG synthesis, levels of prostaglandins are virtually undetectable.

In contrast, Willoughby and others (1973) have elaborated the hypothesis that prostaglandins are largely activated at an inflammatory site by migrating leucocytes. The use of selective inhibitors of histamine, 5-HT and kininogen by this group has demonstrated a 'prostaglandin phase' in carrageenan-induced inflammation, and this phase is dependent upon both an intact complement system and the migration of cells into the inflamed site. Such evidence has however been questioned by Brocklehurst and Dawson (1974), who point out that

in this model the phase of acute inflammation is over before any significant amount of PG is detected and suggest that prostaglandins may be a product of the reaction rather than a cause of the swelling. The concurrent appearance of lysosomal enzymes and prostaglandins in the exudate suggests that dead or damaged cells are the source of both.

A third possibility, proposed by Glatt, Peskar and Brune (1974) from their studies in chickens, is that platelets are the major source of PG-like material released into the joint fluid by urate crystals. An important physiological stimulus for platelet aggregation is adhesion to collagen; at an inflammatory site platelets may adhere to collagen of the subendothelium at points where the endothelium is either damaged or missing, resulting in aggregation of fresh platelets arriving at the site (Baumgartner, 1974). When platelets undergo 'second phase' aggregation (also known as the platelet release reaction), large amounts of prostaglandins, their cyclic endoperoxide precursors and the recently discovered thromboxanes are formed (Smith and Willis, 1971; Willis, 1973; Hamberg, Svennson and Samuelsson, 1975). A recent finding of great interest (Moncada and others, 1976) is that microsomes prepared from rabbit or pig aortas are able to convert PGG_2 or PGH_2 to an unstable substance ('PGX') that inhibits human platelet aggregation. It is suggested that generation of PGX by vessel walls could be the biochemical mechanism underlying their unique ability to resist platelet adhesion.

The work presented in this chapter was carried out in order to determine PG synthetase levels in peripheral and synovial fluid leucocytes, and to compare the action of the aspirin-like drugs on the PG synthetase of leucocytes with the results obtained using R.A. synovial PG synthetase. Furthermore, as it is not possible to carry

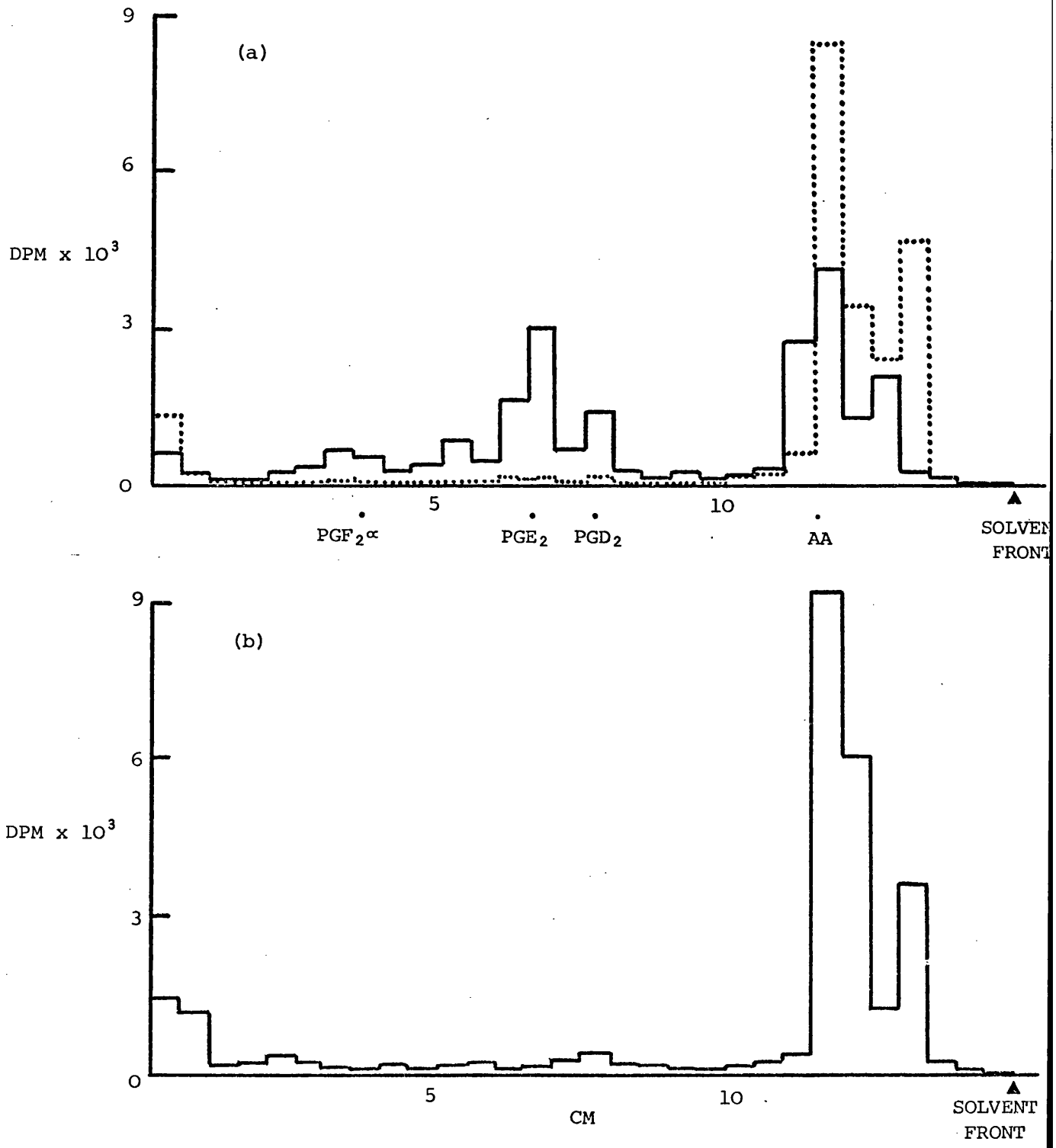
out serial estimations of enzyme activity in human synovium after drug therapy, or to prepare tissue slices for *in vitro* studies, it was also hoped that studies using peripheral leucocytes, both *in vitro* and *in vivo*, might confirm the finding in the previous chapter that aspirin and indomethacin differ in the nature of their action on PG synthetase.

5.2 Metabolism of ^{14}C-arachidonic acid by human leucocyte microsomes

(i) 'Total' leucocyte preparation

The microsomal fraction of a 'total' leucocyte preparation from a healthy volunteer, obtained as described in section 2.3, was incubated with ^{14}C-arachidonic acid (100nCi) for 1 hour at 37^{o}c, and the formed products, together with unchanged substrate, extracted with diethyl ether and separated using thin-layer chromatography (see sections 2.4 and 2.5). Fig 5.1 (a) shows that such a microsomal preparation is capable of extensive arachidonic acid metabolism *in vitro*. The three peaks seen with R_f values of 0.24, 0.44 and 0.51 were coincident with authentic markers of $PGF_2\alpha$, PGE_2 and PGD_2 respectively and were tentatively identified as such. The addition of indomethacin (10^{-5}M) to an identical incubate virtually abolished the production of these three peaks of radioactivity, suggesting that these compounds are indeed products of PG synthetase. A peak running ahead of arachidonic acid with an R_f value of 0.87 was not abolished by the addition of indomethacin and is possibly a hydroxy-acid formed by the action of a lipoxygenase upon the ^{14}C-arachidonic acid. Interest in this peak was not pursued but could presumably be investigated with the use of a lipoxygenase inhibitor such as eicosatetraynoic acid (TYA). Similar chromatographic separations were obtained when the other solvent systems described in section 2.6 were employed.

Fig 5.1 Metabolism of ^{14}C-arachidonic acid by a microsomal fraction of normal human leucocytes



(a) A 'total' leucocyte preparation was used to prepare a microsomal fraction which was incubated with ^{14}C-arachidonic acid (100nCi) at 37°c for 1 hour (———). Products running in the positions of $PGF_2\alpha$, PGE_2 and PGD_2, shown below the scan, were abolished by the addition of indomethacin (10^{-5}M) to an identical incubate (·····).

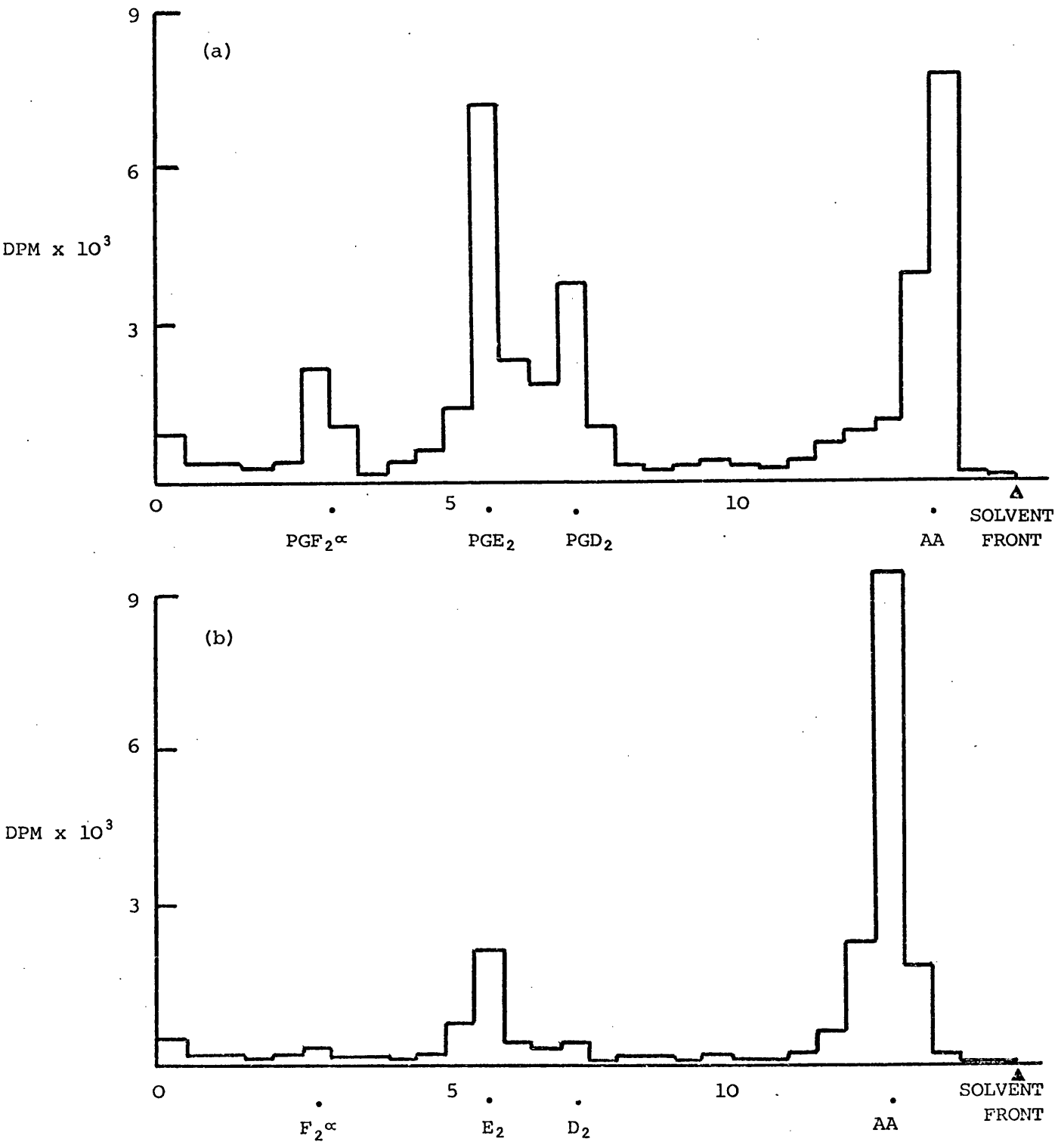
(b) Leucocyte preparation from person who had taken aspirin (600mg) 30 mins before venepuncture.

A microsomal fraction was also obtained from the total leucocyte preparation of a healthy volunteer who had ingested aspirin (600mg) one hour before blood was taken. When incubated with ^{14}C-arachidonic acid this preparation produced only a tiny amount of product running in the prostaglandin region of the plate, Fig 5.1(b), though the 'hydroxy-acid' peak running ahead of the substrate was still present. This finding provided an indication that aspirin could irreversibly inhibit peripheral leucocyte PG synthetase in agreement with the results obtained in the previous chapter using R.A. synovial PG synthetase. Further effort was directed towards determining which cells were the principal source of PG synthetase in such 'total' leucocyte preparations.

(ii) Platelets

Peripheral blood was obtained from several healthy volunteers and a platelet microsomal fraction prepared from each sample as described in section 2.3. When incubated with ^{14}C-arachidonic acid (100nCi) at 37^{o}c for 1 hour these preparations produced extensive metabolism of the added substrate, a typical thin-layer separation of the incubation products being shown in Fig. 5.2(a). The principal product, representing 37% conversion of the substrate, was tentatively identified as ^{14}C-PGE_2. Smaller peaks, corresponding to authentic markers of $PGF_{2\alpha}$ and PGD_2, represented 11% and 16% substrate conversion respectively. For eight subjects the mean conversion of substrate to ^{14}C-PGE_2 was 31.5%, representing a production of 4,220 pmol/mg/hour, though the range was large (700-10,500 pmol/mg/hour). These values are at least an order of magnitude higher than those found for R.A. synovial microsomes in section 4.5 (mean values 171-256 pmol E_2 produced /mg/hour). However, in three volunteers who had taken aspirin (600 mg) one hour before blood samples were taken (see section 5.8 for details) the mean production of ^{14}C-PGE_2 in vitro

Fig. 5.2 Metabolism of ^{14}C-arachidonic acid by microsomes from (a) normal human platelets and (b) normal human PMN's.



Microsomal fractions from normal human platelets or polymorphonuclear leucocytes were prepared as described in section 2.3 and incubated with ^{14}C-arachidonic acid in 0.1M tris-acetate buffer, pH8.0, at 37^{o}c for 1 hour. The positions of authentic markers are shown below the scan.

by their platelet microsomes was 191 pmol/mg/hour, range 157-226 pmol/mg/hour), again confirming that administration of aspirin *in vivo* largely abolished subsequent microsomal PG synthetase activity *in vitro*.

No qualitative or quantitative differences were found between platelet microsomes obtained from patients with rheumatoid arthritis and those prepared from healthy volunteers. Microsomes from eight patients receiving indomethacin therapy (175-225 mg/day) produced a mean substrate conversion to ^{14}C-PGE_2 of 37.3%, representing 3,920 pmol/mg/hour, range 920-9,750 pmol/mg/hour. However in four patients receiving aspirin therapy (2.4-3.6 g/day) the mean conversion of substrate to ^{14}C-PGE_2 was only 1.4%, representing a mean production of only 78 pmol/mg/hour.

(iii) PMN's

Polymorphonuclear leucocytes were prepared from the peripheral blood of six healthy volunteers and a microsomal fraction from each prepared as described in section 2.3. Fig 5.2(b) shows the thin-layer separation of the products of an incubate containing PMN microsomes and ^{14}C-arachidonic acid. The mean conversion of the substrate to the radioactive product running in the position of authentic PGE_2 was 5.1%, representing a mean production of ^{14}C-PGE_2 of 255 pmol/mg/hour, range 96-510 pmol/mg/hour.

PMN microsomes from six patients with R.A. who were receiving indomethacin therapy (175-225mg/day) produced a mean substrate conversion to the product identified as ^{14}C-PGE_2 of 25.1%. However when the figure for production of PGE_2 was calculated on the basis of microsomal protein content, the mean value (371 pmol/mg/hour) was not significantly different from that found with the preparations

from the healthy volunteers. PMN microsomes from three patients with R.A. who were receiving aspirin therapy (2.4-3.6 g/day) showed a mean of only 1.7% conversion of ^{14}C-arachidonic acid to ^{14}C-PGE_2, a mean production of only 28 pmol/mg/hour, range 21-35 pmol/mg/hour.

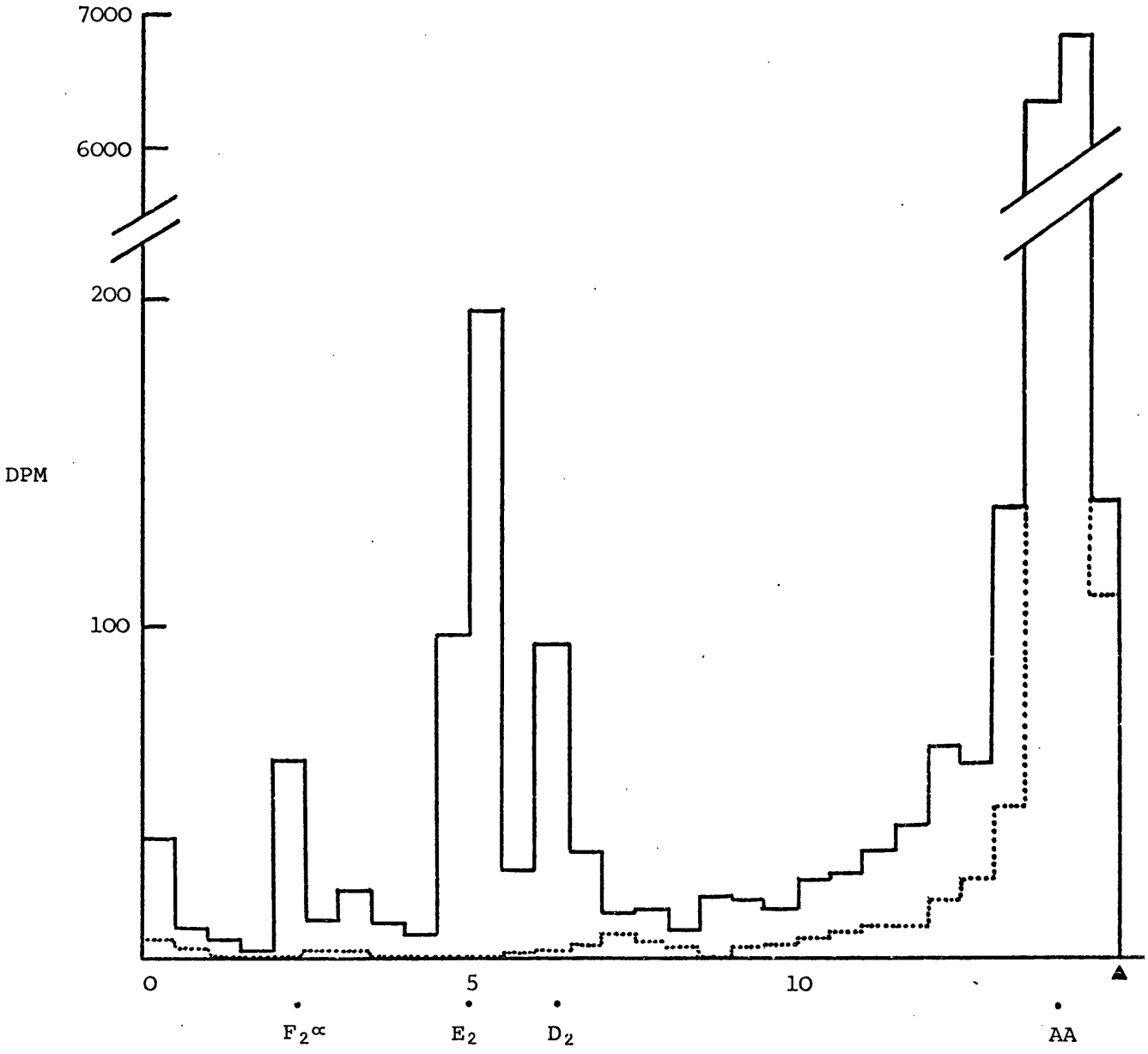
(iv) Lymphocytes

Lymphocytes were prepared from the peripheral blood of seven healthy volunteers and a microsomal fraction obtained from each as described in section 2.3. Incubation of these microsomal fractions with ^{14}C-arachidonic acid produced no detectable prostaglandin synthesis in any sample, even when, in two cases, 50ml of whole blood was used for the cell preparation. This finding is in agreement with the report of Ferraris and De Rubertis (1974) that lymphocytes in culture produce only tiny amounts of prostaglandins in response to stimulation by either antigens or mitogens. Unfortunately no studies were possible on lymphocytes prepared from patients with rheumatoid arthritis though the finding of an active preparation would be surprising in view of the findings cited above.

(v) Synovial fluid leucocytes

Fig. 5.3 shows the scan obtained when the microsomal fraction of a 'total' leucocyte preparation from the synovial fluid of a patient with R.A. was incubated with ^{14}C-arachidonic acid (100 nCi) at 37^{o}c for 1 hour, (0.24 mg protein per incubation). Although sharp peaks of activity were seen in the positions of $PGF_2\alpha$, PGE_2 and PGD_2 these represented only 0.7%, 2.9% and 1.3% substrate conversion respectively. Addition of indomethacin (10^{-5}M) to an identical incubation abolished these peaks, providing good evidence that they were produced by the action of PG synthetase on the labelled substrate. Production of prostaglandins by several other synovial fluid leucocyte microsome preparations, both with and

Fig 5.3 Metabolism of ^{14}C-arachidonic acid by microsomes from R.A. synovial fluid leucocytes



A microsomal fraction prepared from rheumatoid synovial fluid leucocytes was incubated with ^{14}C arachidonic acid (100nCi) in 0.1M tris-acetate buffer, pH 8.0, at 37°c for 1 hour. The positions of authentic markers are shown below the scan. The conversion of ^{14}C-arachidonic acid to ^{14}C-PGE_2 was less than 3%.

——— Control

..... $+10^{-5}$M Indomethacin

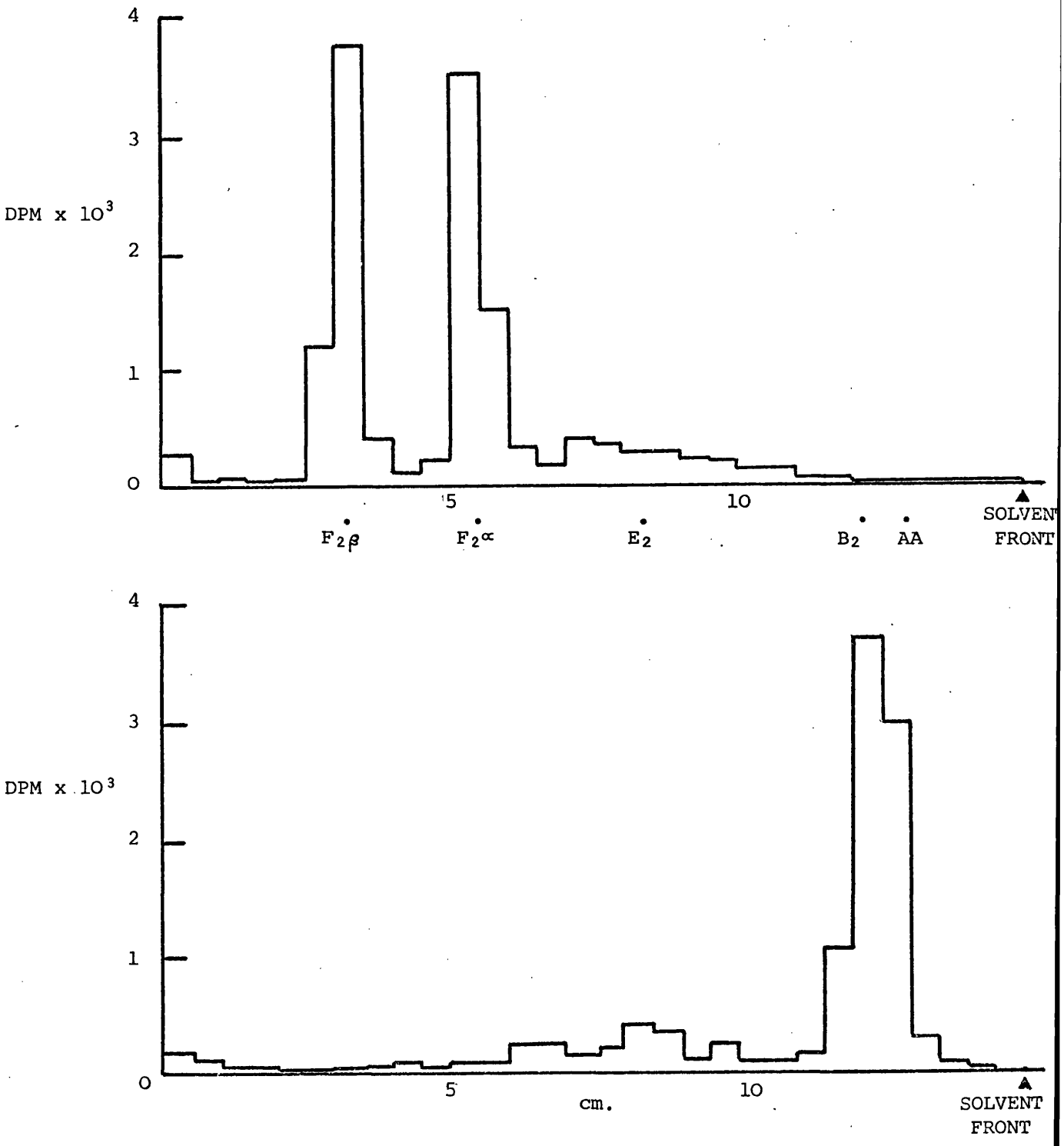
without hyaluronidase pre-incubation, was barely detectable, suggesting that synovial fluid leucocytes are not a good source of PG synthetase. This is perhaps a little surprising in view of the finding of Higgs, McCall and Youlten (1975) that the levels of PG synthetase found in PMN's undergoing phagocytosis are increased severalfold over the levels in resting cells.

Taken together, the results presented above suggest that platelets contain by far the greatest biosynthetic potential for PG synthesis and preliminary in vivo results with aspirin and indomethacin confirm the findings discussed in the previous chapter that aspirin may be unique in being an irreversible inhibitor of PG synthetase. Further efforts therefore were directed towards the study of the interaction between aspirin or indomethacin and human platelets as a 'model' cell for the study of PG synthetase.

5.3 Chemical conversion of PGE_2

Before proceeding with further studies it was considered desirable to confirm that the principal product of arachidonic acid metabolism produced by human platelet microsomes under the incubation conditions described was in fact PGE_2. Fig. 5.4 shows that the radioactive product tentatively identified as ^{14}C-PGE_2 behaved exactly as expected, producing virtually identical scans to ^{14}C-PGE_2 isolated from R.A. synovial microsome incubations in Fig. 3.2. Thus treatment of the isolated product with sodium borohydride produced equal amounts of activity running in the positions of $PGF_2\alpha$ and $PGF_2\beta$, and sodium hydroxide treatment produced a single peak of activity running in the position of PGB_2. This, together with chromatographic identification in three different systems provides good evidence that the product is in fact PGE_2. (This might be expected as the presence of GSH in

Fig. 5.4 Chemical conversion of formed ^{14}C-PGE$_2$ with sodium borohydride or sodium hydroxide



^{14}C-PGE$_2$ formed from the incubation of human platelet microsomes with ^{14}C-arachidonic acid was isolated by thin-layer chromatography and treated with either NaBH$_4$ or NaOH. Re-chromatography of the extract showed conversion to an equal mixture of PGF$_2\alpha$ and PGF$_2\beta$ upon NaBH$_4$ treatment (upper scan) and conversion to PGB$_2$ with NaOH (lower scan).

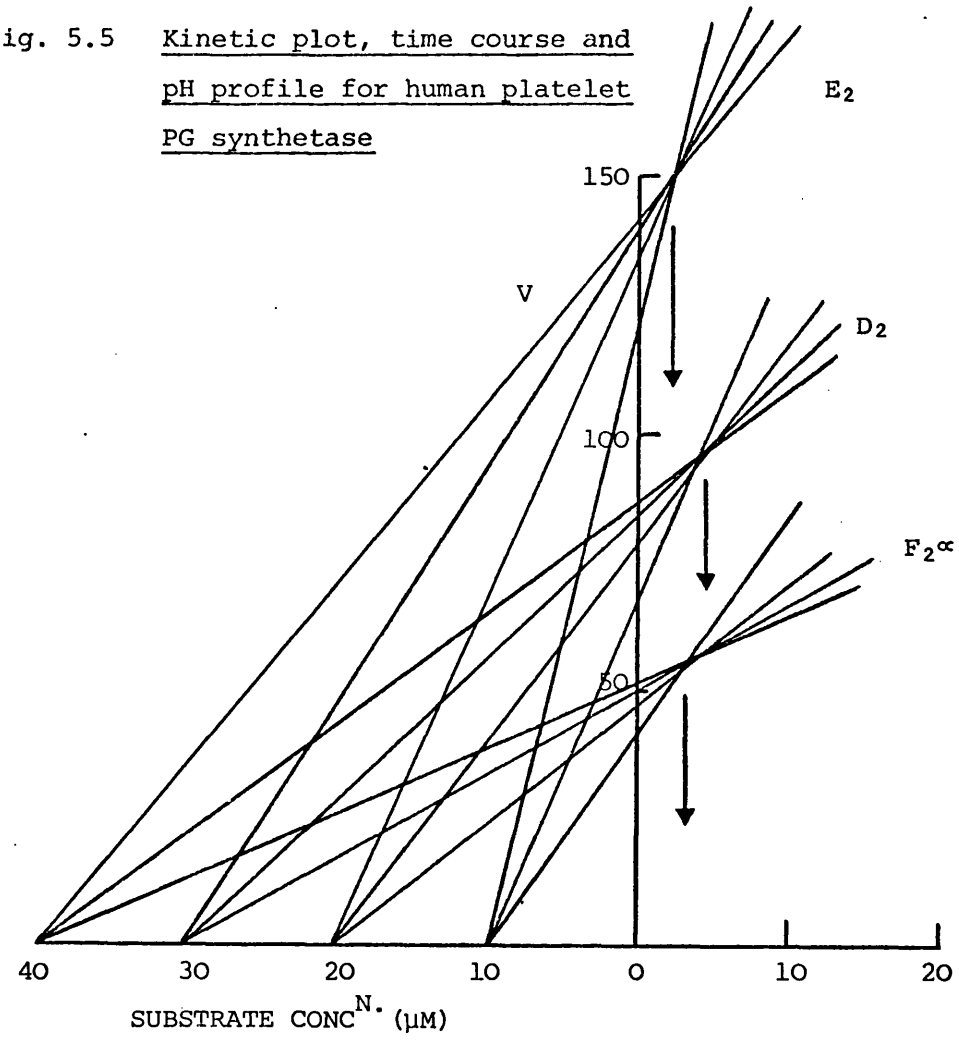
incubations is known to promote PGE synthesis at the expense of other prostaglandins).

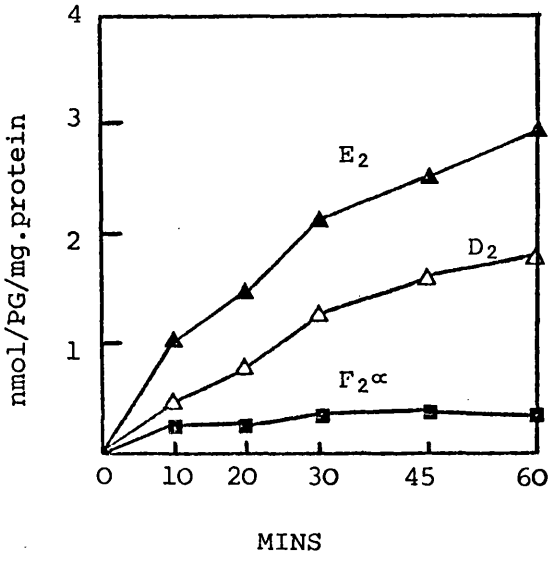
5.4 Biochemistry of human platelet PG synthetase

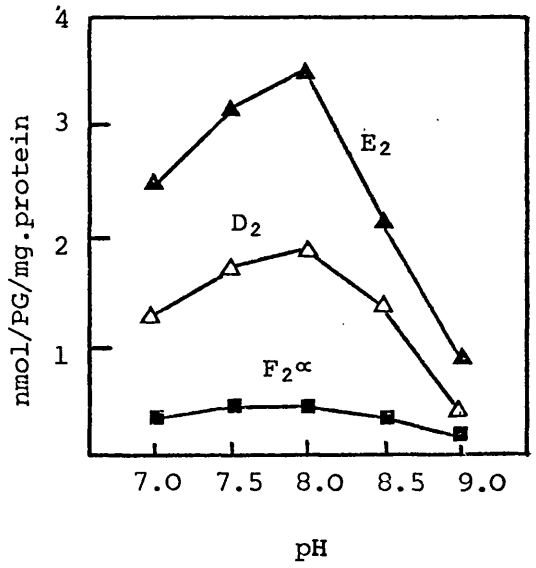
Fig. 5.5 shows a kinetic plot, time course of PG production, and pH profile for human platelet PG synthetase when incubated *in vitro* with ^{14}C-arachidonic acid. The enzyme has a pH optimum at pH 8.0 and in this respect is indistinguishable from the synthetase prepared from human R.A. synovial tissue (see Fig. 3.6) although the peak is somewhat sharper for the platelet enzyme. As found for R.A. synovial PG synthetase the time course of PG production by human platelet microsomes is linear for the first 30 minutes of the incubation. After this time the production of prostaglandins increases further but is no longer linearly related to time. An incubation time of 1 hour was chosen for all incubations except those used to construct a kinetic plot, for which an incubation time of 30 minutes was chosen.

When such a kinetic plot was constructed a marked difference was found between the calculated K_m values and those measured for the synovial enzyme. Thus the K_m values for human platelet PG synthetase, calculated on the basis of PGE_2, $PGF_{2\alpha}$ or PGD_2 production were 2.4, 3.2 and 4.4μM respectively, compared with values for R.A. synovial PG synthetase of approximately 12μM. However, as the microsomal preparations used in these studies are grossly impure preparations of the enzymes such results should perhaps be interpreted with caution. As discussed in detail by Youdim and Woods (1975) even the K_m value obtained using a highly purified enzyme *in vitro* may be inapplicable to the situation *in vivo*.

Fig. 5.5 Kinetic plot, time course and pH profile for human platelet PG synthetase







Calculated Km values were 2.4μM for PGE_2 formation, 3.2μM for $PGF_{2\alpha}$ and 4.4μM for PGD_2.

5.5 Inhibition of human platelet PG synthetase by aspirin-like drugs in vitro

When added to active microsomal preparations of human platelet PG synthetase, both aspirin and indomethacin produced the expected dose-related inhibition (Fig. 5.6). Calculated from the dose of each drug required to produce 50% inhibition of PG biosynthesis, and expressed on a molar basis, indomethacin was approximately 150 times more potent than aspirin. The production of PGE_2 and $PGF_{2\alpha}$ was equally affected, and no evidence was found for 'selective' inhibition by either drug.

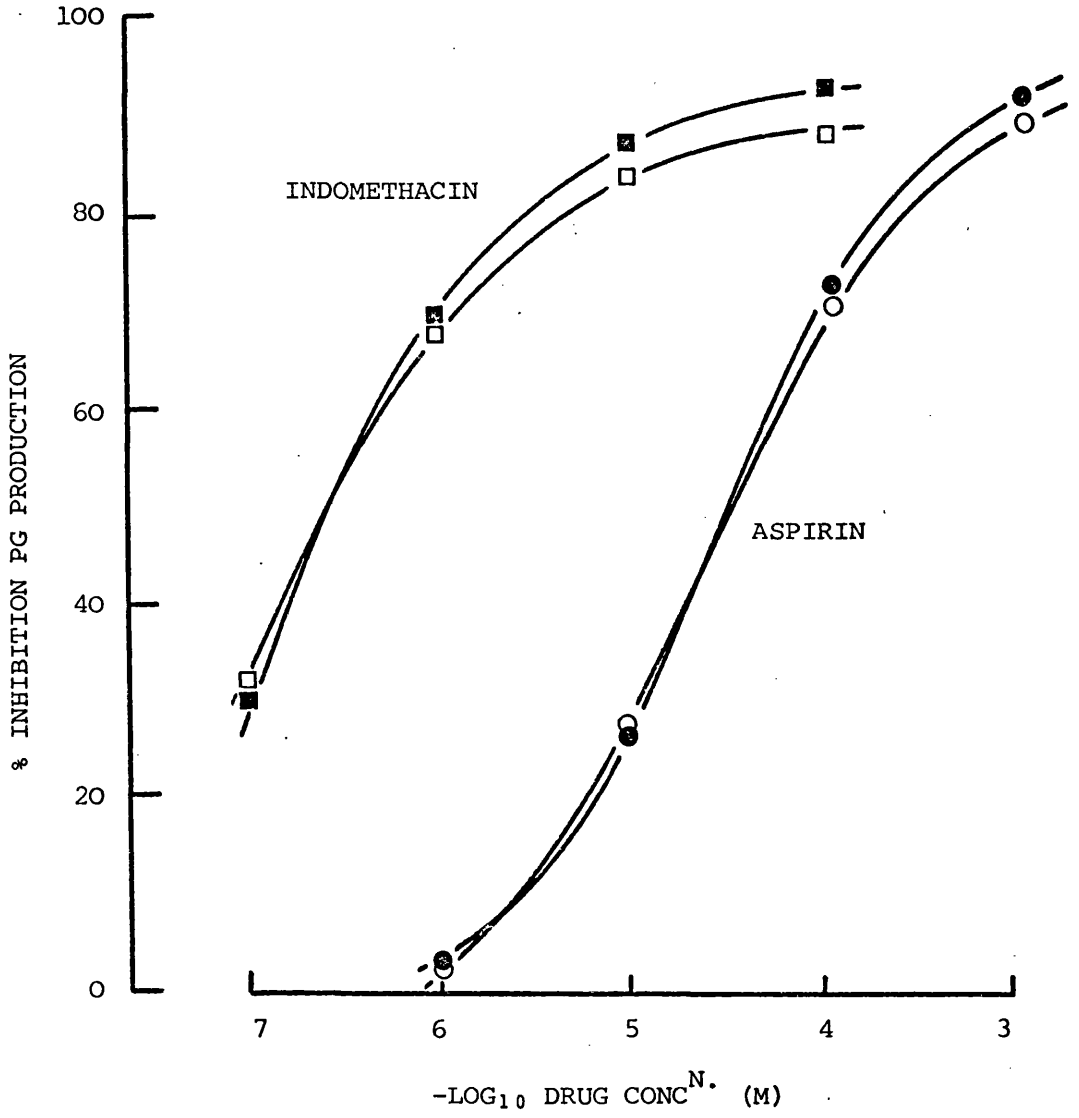
Of other aspirin-like drugs examined in this system, similar potencies were found to those measured using R.A. synovial tissue as the source of the enzyme. An exception was azopropazone which was twice as potent as aspirin as an inhibitor of R.A. synovial PG synthetase *in vitro*, but only $\frac{1}{4}$ - $\frac{1}{5}$ as potent as aspirin on the enzyme prepared from human platelets.

5.6 Pre-incubation of whole platelets with aspirin or indomethacin

Aliquots of platelet-rich plasma were incubated with either aspirin or indomethacin over the concentration range 10^{-6}M - 10^{-3}M, at 37° for 30 min. with shaking. The platelet pellet obtained by centrifugation at 2500g for 5 min. was resuspended in Tyrode's solution (5.0 ml), incubated at 37° for a further 10 min. and the platelet pellet again obtained by centrifugation. The preparation of the microsomal fraction and measurement of PG synthetase activity were then carried out as described in sections 2.3-2.5.

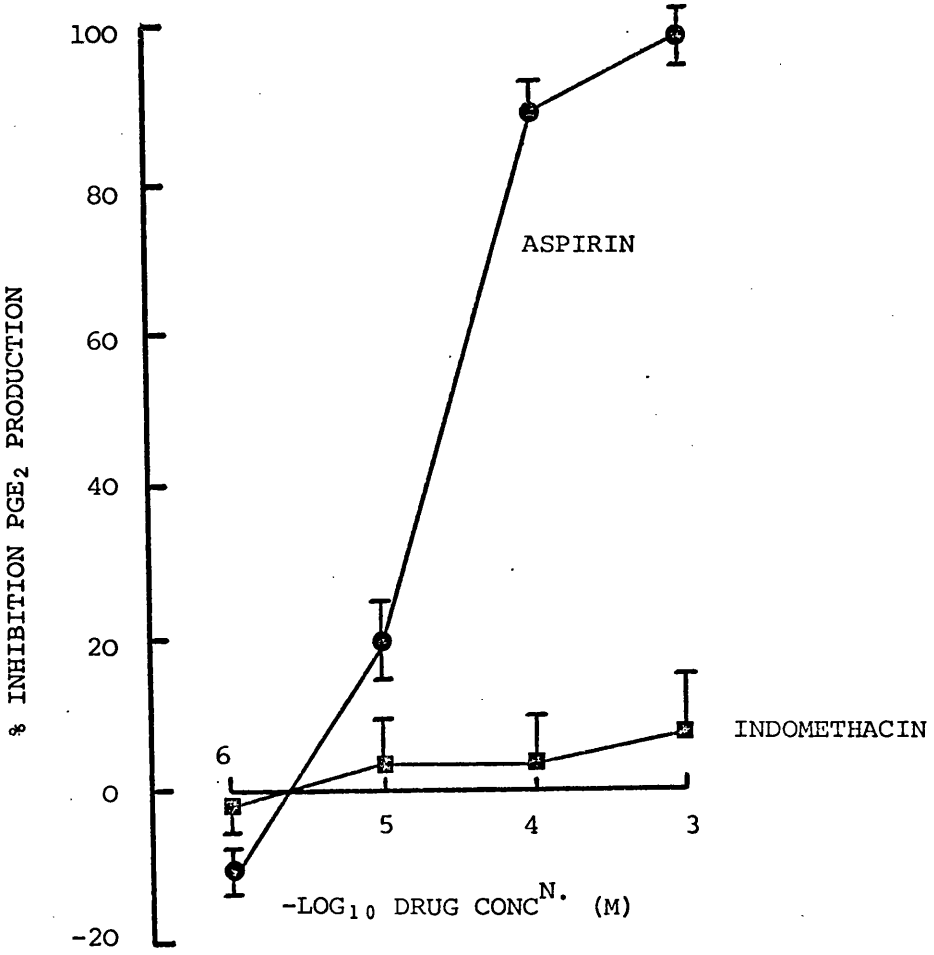
The results are presented in Fig. 5.7 and show a marked difference between the two drugs. Thus whilst exposure to aspirin at

Fig. 5.6 Inhibition of human platelet microsomal PG synthetase by aspirin and indomethacin



Human platelet microsomes were incubated with ^{14}C-arachidonic acid as described in sections 2.4 and 2.5. Production of PGE_2 (closed symbols) and $PGF_{2\alpha}$ (open symbols) was inhibited in a dose-related manner by the addition of aspirin (●—●, ○—○) or indomethacin (■—■, □—□). Points represent the mean of duplicate estimation.

Fig. 5.7 Inhibition of human platelet microsomal PG synthetase after pre-incubation of whole platelets with aspirin or indomethacin



Whole human platelets were pre-incubated with either aspirin or indomethacin at 37°c. for 30 min. over the concentration range 10^{-6}M - 10^{-3}M and the PG synthetase activity of the subsequent microsomal fraction from each incubation measured as described in sections 2.4 and 2.5. Whereas aspirin (●——●) produced a dose-dependent inhibition, indomethacin (■——■) had little or no effect. Points are shown with the standard error of the means for 4 estimations.

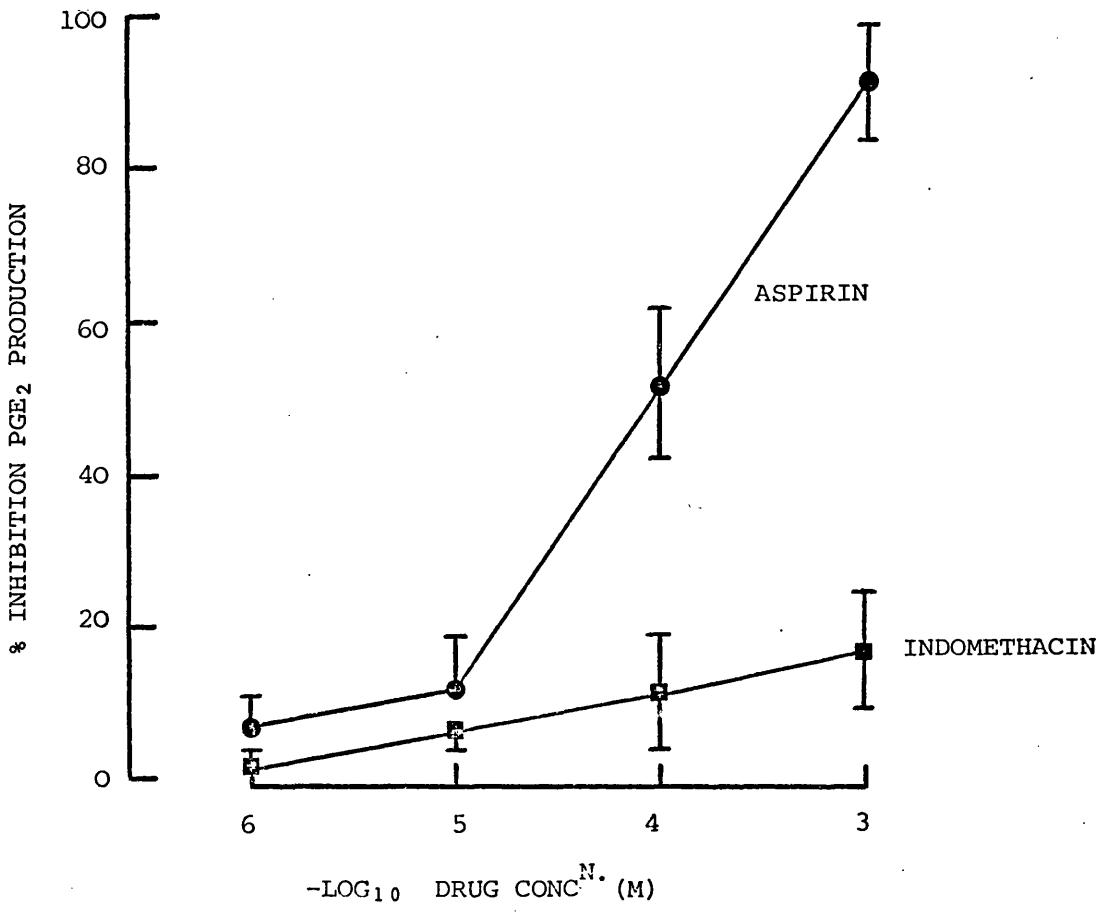
a concentration of 10^{-4}M was capable of producing 80-90% inhibition of PG synthetase, indomethacin had little or no effect, even a concentration of 10^{-3}M producing less than 10% inhibition of subsequent microsomal PG synthetase activity. The apparent stimulation of PG synthesis by aspirin at 10^{-6}M is not readily explained, though similar phenomena have been reported in other systems (Brocklehurst and Dawson, 1974; Stone, Mather and Gibson, 1975). The most potent inhibitor of PG synthetase found in these studies, flurbiprofen, was found, like indomethacin to be incapable of permanently inhibiting microsomal PG synthetase by pre-incubation with whole platelets. The use of flurbiprofen coupled to sepharose for the affinity chromatographic purification of PG synthetase (Smith, 1975) implies that the interaction between the enzyme and flurbiprofen (or at least its amide derivative) must be reversible as an active fraction is recovered upon elution with a solution of flufenamic acid.

5.7 Pre-incubation of human platelet microsomes with aspirin or indomethacin

Human platelet microsomes were re-suspended in Tyrode's solution and aliquots incubated with either aspirin or indomethacin over the concentration range 10^{-6}M - 10^{-3}M, at 37^{o} for 30 min. with shaking. The suspension was then layered onto tris-acetate buffer (0.1M, pH 7.4) containing sucrose (0.25M) and a microsomal pellet obtained by centrifugation at 10^{5}g for 1 hr. The PG synthetase activity of each microsomal pellet was then measured as described in sections 2.4 and 2.5.

The results are shown in Fig. 5.8 and confirm the findings described above using whole platelets. Thus whilst aspirin produced a dose-related inhibition of PG synthetase activity of similar potency

Fig. 5.8 Inhibition of human platelet microsomal PG synthetase after pre-incubation of platelet microsomes with aspirin or indomethacin



Human platelet microsomes were pre-incubated with either aspirin (●——●) or indomethacin (■——■) at 37°c for 30 min. over the concentration range 10^{-6}M - 10^{-3}M and the PG synthetase activity of the washed microsomes measured as described previously. Points are shown with the standard error of the means for 4 estimations.

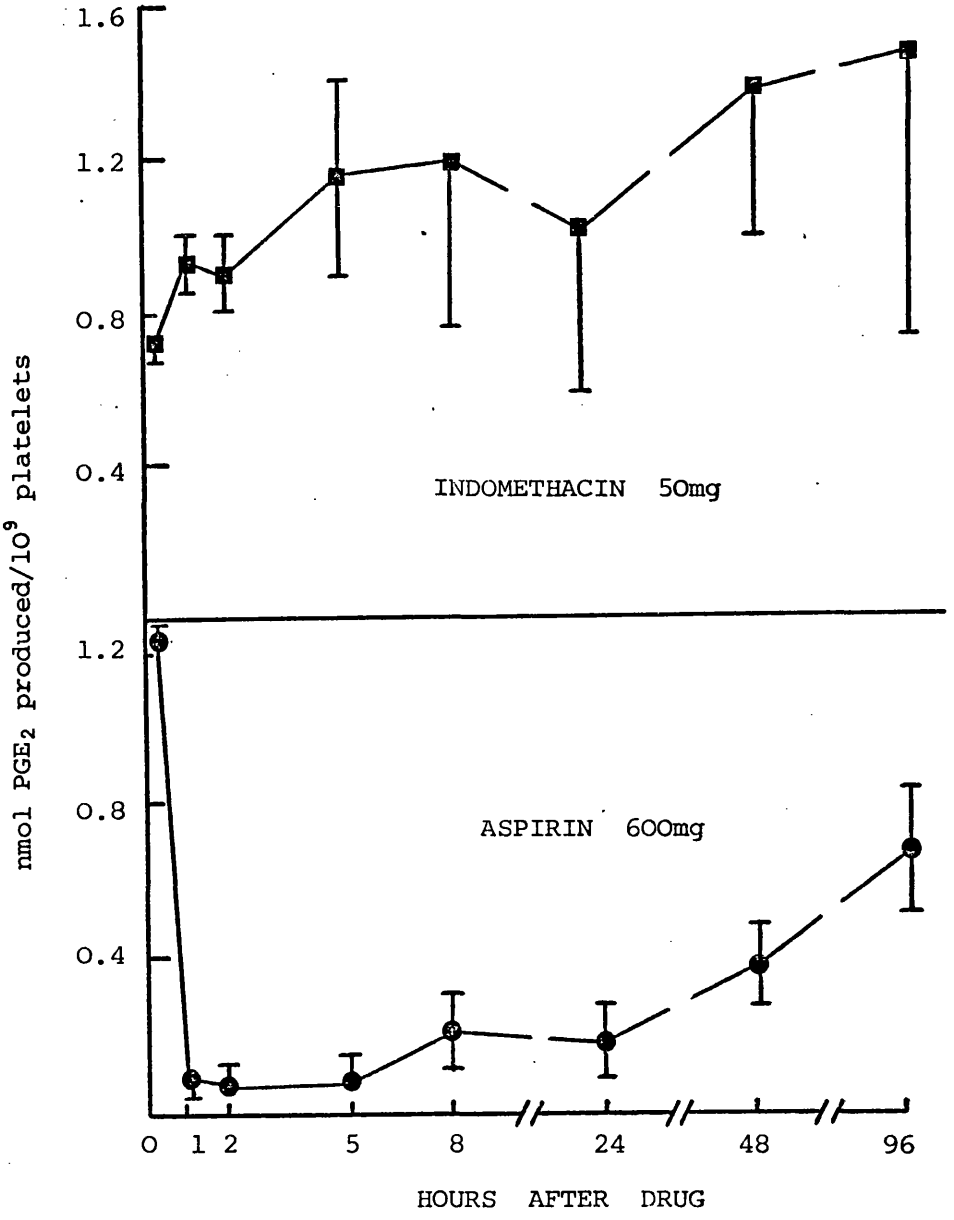
to that found when present in an incubation mixture containing platelet microsomes and ^{14}C-arachidonic acid, indomethacin was considerably less potent than aspirin in producing inhibition of PG synthetase by pre-incubation. As indomethacin is approximately 150 times more potent than aspirin as a PG synthetase inhibitor when present in an incubation mixture containing platelet microsomes and ^{14}C-arachidonic acid (Fig. 5.6), these findings strongly suggest, as found with R.A. synovial PG synthetase, that indomethacin is a reversible inhibitor of human platelet PG synthetase, whereas the inhibition produced by aspirin is irreversible.

5.8 The effect of aspirin and indomethacin in vivo

Three healthy male volunteers who had received no medication of any kind for at least two weeks beforehand were given indomethacin (50mg) orally, and blood samples obtained over the following 96 hr, including a pre-drug, basal sample. Three weeks later the procedure was repeated with the subjects taking aspirin (600 mg). Platelet-rich plasma was prepared from each sample, a platelet count performed, and the PG synthetase activity of the microsomal fraction measured as described above, using a constant number of platelets from each subject. Plasma levels of indomethacin were measured by the method of Hucker and others (1966), and salicylate by the method of Øie and Frislid (1971).

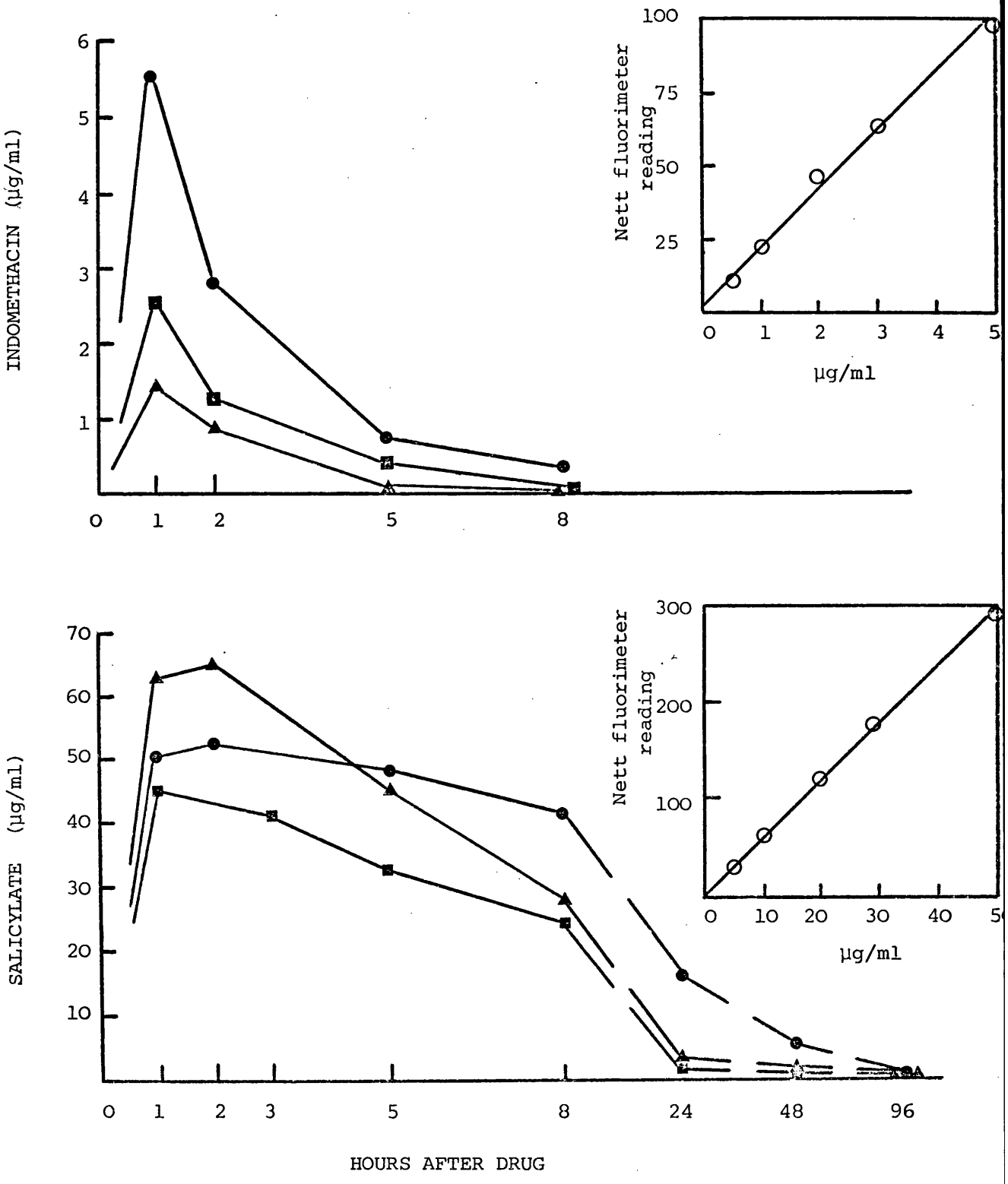
Administration of aspirin (600 mg) to 3 volunteers produced a prompt fall in platelet microsomal PG synthetase activity *in vitro* to less than 15% of basal levels, and this blockade lasted for several days (Fig. 5.9, lower trace). As platelets are devoid of a nucleus and contain little or no DNA they are capable of very limited *de novo* enzyme synthesis (Steiner, 1970). The time course of the

Fig. 5.9 Effect of orally administered aspirin or indomethacin on subsequent microsomal PG synthetase activity from human platelets



3 healthy volunteers each took indomethacin (50mg) orally. Blood samples were taken at intervals over the following 96 hours, platelets prepared from each sample and the PG synthetase activity of the microsomal fraction from each one measured as described previously. Three weeks later the procedure was repeated with the volunteers taking aspirin (600mg). Points are shown with the standard error of the mean.

Fig. 5.10 Plasma levels of indomethacin and salicylate after oral ingestion of indomethacin or aspirin



Indomethacin levels were measured using the method of Hucker and others (1966) and the method of Øie and Frislid (1971) was used to determine total salicylate levels. Calibration lines for each measurement are shown in the insets.

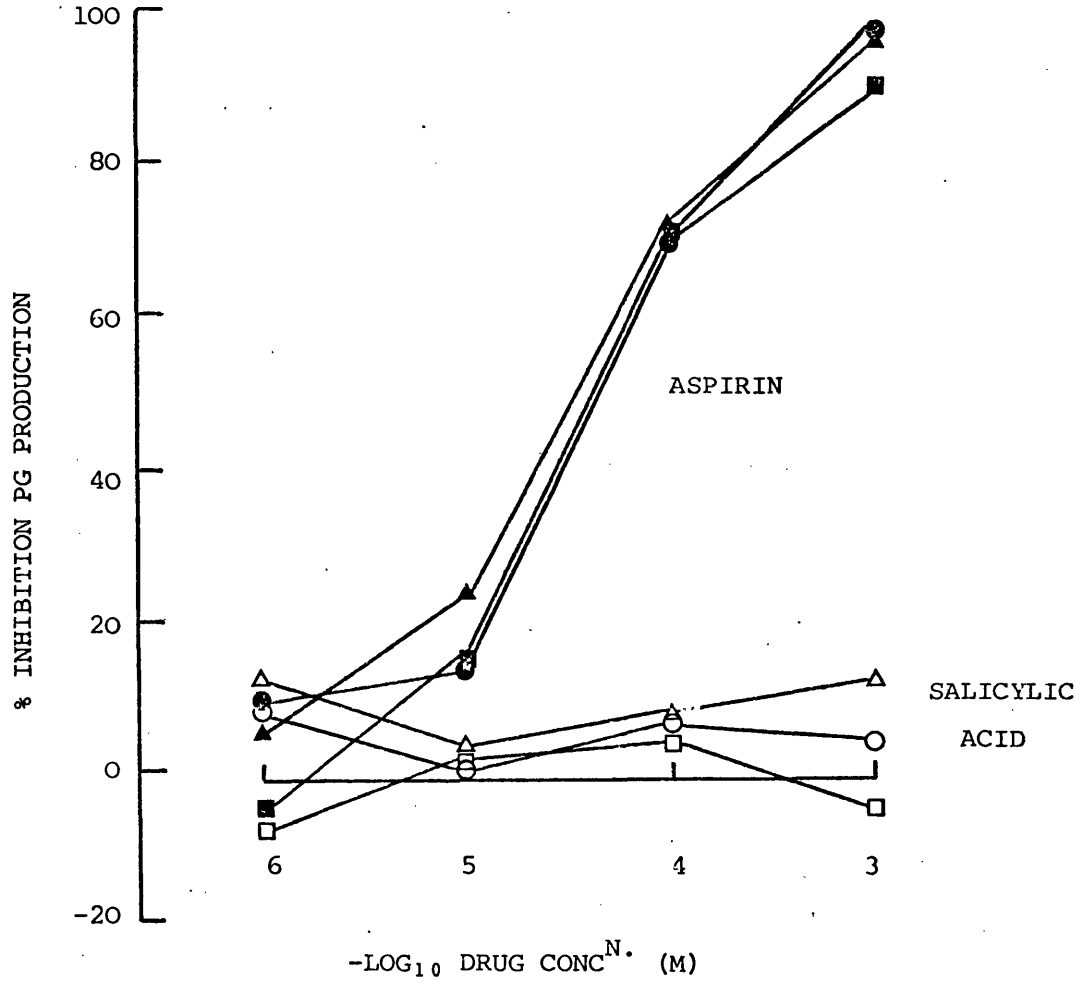
inhibition of platelet PG synthetase by aspirin is thus effectively a measure of platelet turnover.

By contrast ingestion of indomethacin (50 mg) produced no reduction of microsomal PG synthetase activity in two individuals, and in the third subject brought about a stimulation of activity which at 24 hr. after the drug had been taken was more than twice the basal level. This accounts for the large standard errors shown in the upper trace of Fig. 5.9.

Fig. 5.10 shows the plasma levels of either indomethacin or total salicylate of the three volunteers in the above experiment, as determined by standard fluorimetric techniques. As expected, indomethacin produced maximum plasma concentrations one hour after oral ingestion and was largely cleared from the blood at five hours (Rothermich, 1966). In contrast, the concentration of total salicylate in the plasma was more sustained after the single oral dose of 600 mg aspirin, lying in the range 20 - 40 µg/ml at 8 hr. after taking the drug, and was still detectable at 48 hr. in one subject. It should be noted however that although the half-life of plasma salicylate in man is of the order of 6 hours, aspirin is rapidly de-acetylated in plasma and has a half life of only 20-30 minutes (Levy, 1963). Significant concentrations of aspirin are unlikely to be present in the blood more than 2 hours after a single oral dose (Lester, Lolli and Greenberg, 1946).

The possibility that the prolonged inhibition of platelet PG synthetase seen after administration of aspirin <u>in vivo</u> could be due to salicylic acid, or one of its metabolites, was investigated by testing the potency of these compounds as inhibitors of platelet PG synthetase <u>in vitro</u>. Fig. 5.11 shows that although aspirin produced the expected dose-related inhibition of platelet PG synthetase,

Fig. 5.11 Inhibition of human platelet PG synthetase in vitro by aspirin and salicylic acid



Human platelet microsomes (0.14mg protein/incubation) were incubated in 0.25M sucrose/0.1M tris-acetate buffer, pH 8.0, with ^{14}C-arachidonic acid (100nCi) for 1 hour at 37°c. Production of ^{14}C-PGE$_2$ (●——● , ○——○), ^{14}C-PGF$_{2\alpha}$ (■——■ , □——□), and ^{14}C-PGD$_2$ (▲——▲ , △——△) was inhibited in a dose-related manner by aspirin (closed symbols) though salicylic acid (open symbols) was virtually inactive in this respect.

salicylic acid was inactive in this respect. Similarly, as found with the enzyme prepared from R.A. synovial tissue, the four dihydroxybenzoic acids were also inactive. It can reasonably be concluded therefore that aspirin exerts its prolonged inhibition of platelet PG synthetase by an irreversible action on the enzyme. As discussed in the following chapter, much evidence now exists to suggest that this may be brought about by acetylation of the cyclo-oxygenase component of the enzyme.

5.9 The effect of Cu^{++} and Zn^{++} on human platelet PG synthetase

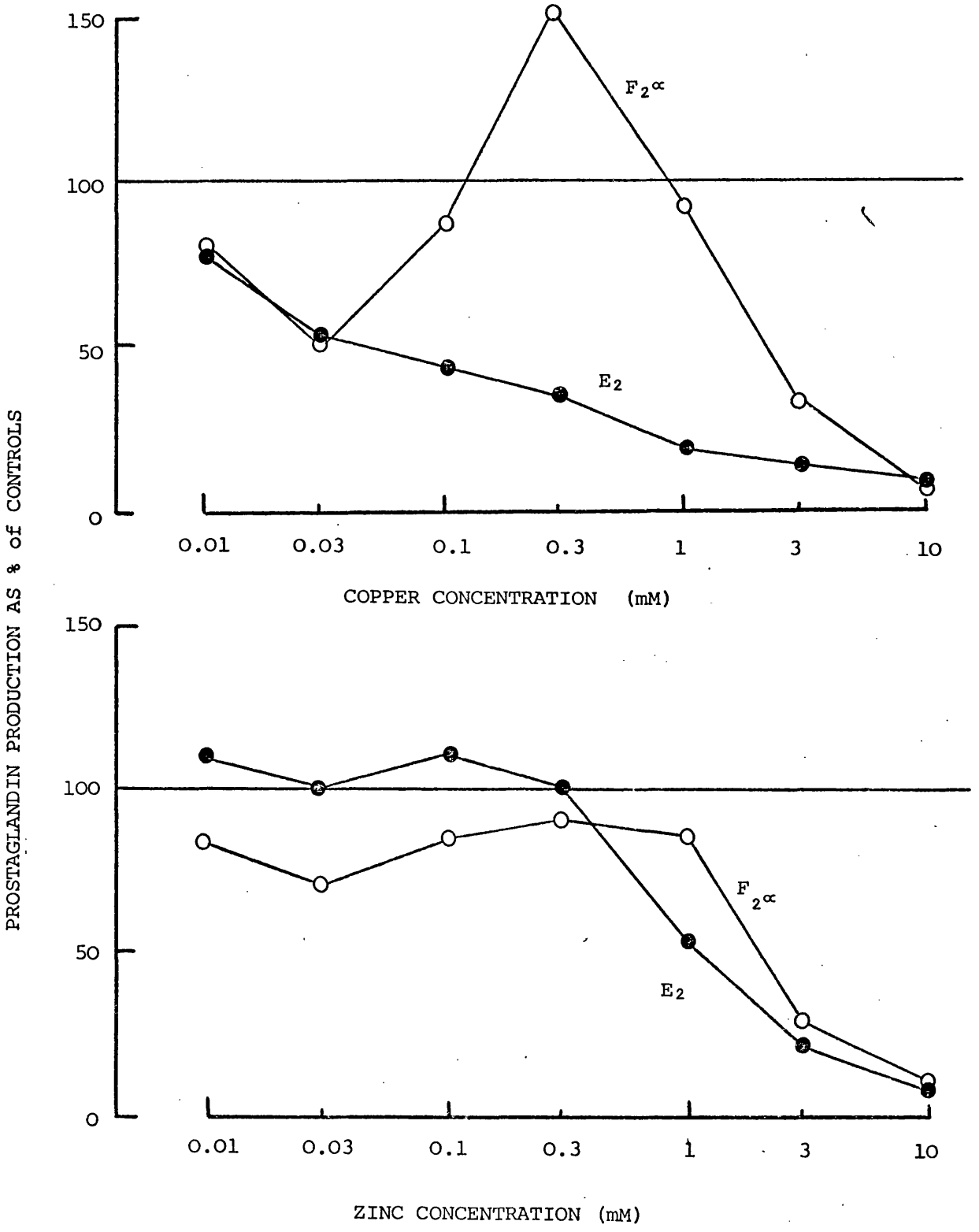
The effect of copper ions on platelet PG synthetase was studied in order to confirm the findings in section 4.4 using the enzyme from R.A. synovial tissue.

When copper ions (as cupric acetate) were added to human platelet synovial microsomes, incubated with ^{14}C-arachidonic acid *in vitro*, over the concentration range 10^{-5}M-10^{-2}M, the results obtained were almost identical to those obtained previously and are shown in Fig. 5.12, upper trace. Whereas the production of PGE_2 was inhibited in a dose-related manner, $PGF_{2\alpha}$ was markedly stimulated at a copper concentration of 3×10^{-4}M. By contrast, the addition of zinc ions (as zinc acetate) produced no stimulation of $PGF_{2\alpha}$ synthesis and inhibition of both PGE_2 and $PGF_{2\alpha}$ only at high concentrations (Fig. 5.12, lower trace). The effect of copper on the enzyme would thus appear to be more specific than a purely chemical one.

5.10 Discussion

The results presented in this chapter were all obtained using PG synthetase prepared from human peripheral or synovial fluid leucocytes. Previous work had shown both platelets (Smith and Willis,

Fig. 5.12 The effect of copper and zinc on human platelet PG synthetase in vitro



Human platelet microsomes were incubated with ^{14}C-arachidonic acid (100nCi) and either copper or zinc acetate added to give a concentration range from 10^{-5}M - 10^{-2}M. Production of ^{14}C-PGE$_2$ was inhibited in a dose-related manner by both copper and zinc (●——●). However, only copper produced a stimulation of ^{14}C-PGF$_2\alpha$ synthesis (O——O) at a concentration of 3 x 10^{-4}M.

1971) and PMN's (Higgs and Youlten, 1972) to be capable of considerable PG synthesis in response to appropriate stimuli. When microsomal fractions were prepared from human peripheral platelets, PMN's and lymphocytes in the present study, by far the greatest levels of PG synthetase activity were found in the platelets. Much lower levels were found in PMN cells and no activity could be demonstrated in the microsomes prepared from human peripheral lymphocytes. Further studies were therefore carried out both in vivo and in vitro on the interaction between the aspirin-like drugs and PG synthetase, with particular attention being given to aspirin and indomethacin, in order to confirm the previous findings obtained using R.A. synovial PG synthetase.

Basic biochemical parameters of human platelet PG synthetase were found to be similar to those described for R.A. synovial PG synthetase, though the different K_m values and somewhat different patterns of products obtained upon incubation with ^{14}C-arachidonic acid in vitro suggest that the activity of synovial microsomes is not due to platelet contamination.

When either whole platelets or platelet microsomes were pre-incubated with aspirin or indomethacin, a marked difference between the two drugs was found in respect of their inhibition of subsequent PG synthetase activity in vitro. Thus, whereas aspirin was able to inhibit the enzyme after a period of pre-incubation prior to washing of the cells or microsomes, indomethacin was a potent inhibitor of PG synthetase activity only when present in incubation mixtures, and had little effect when present in pre-incubation solutions. These findings are in complete agreement with the results presented in Chapter 4, and suggest that the phenomena may be a general one, at least in human cells and tissues.

The finding that administration of aspirin in vivo produces a long-lasting inhibition of human platelet PG synthetase may explain the report that whilst the effect of aspirin in inhibiting both platelet prostaglandin production and platelet aggregation lasts for several days, the effect of indomethacin is short-lived (Kocsis and others, 1973) and can be demonstrated only for as long as an adequate plasma concentration is maintained. A recent report (Jafari and others (1976) confirms the finding in the present study that aspirin produces an inhibition of platelet PG synthetase for the entire life span of the platelet, and suggests that aspirin may also acetylate the developing megakaryocyte prior to platelet release into the circulation.

As discussed in the following chapter the finding that low concentrations of aspirin are able to irreversibly acetylate a platelet particular fraction protein provides strong evidence that this may be its mode of action at the molecular level in inhibiting PG synthetase. Consideration of the structures of other aspirin-like drugs, however, suggests that this is unlikely to be a general property of this group of compounds, as most possess no labile functional group. The results presented have suggested that indomethacin probably does not function as a benzoylating agent in an analogous manner to aspirin, though it would clearly be of interest to investigate this possibility using suitably labelled indomethacin of high specific acitivity.

CHAPTER SIX

STUDIES WITH /ACETYL-^{3}H/ ASPIRIN

6.1 Introduction

The results obtained in the previous two chapters indicate that whereas indomethacin and some of the other aspirin-like drugs are reversible inhibitors of PG synthetase from R.A. synovial tissue and peripheral leucocytes, aspirin itself is an irreversible inhibitor of the enzyme. This action could be brought about by acetylation of the enzyme, possibly at the substrate or cofactor site, as it has previously been shown that aspirin acetylates proteins and other biological substrates. Thus, aspirin at a concentration of 0.5mM acetylates human serum albumin at a single site leading to the formation of ε-N-acetyl lysine residues (Hawkins and others, 1969). At high concentrations (2.0 - 20mM) aspirin acetylates haemoglobin at several sites within the molecule, perhaps through the formation of ε-N-acetyl lysines (De Furia and others, 1974). The drug is also able to acetylate RNA, hormones and various other serum proteins (Pinckard, Hawkins and Farr, 1968) through the mechanisms of the reactions are not well characterised.

The use of /¯acetyl-1-^{14}C¯/ aspirin and /¯acetyl-^{3}H¯/ aspirin (Al-Mondhiry, Marcus and Spaet, 1970; Rosenberg and others, 1971) showed that aspirin acetylates platelet protein, though quantitation was difficult due to the low specific activities of the labelled aspirin used, and characterisation of the labelled protein was not possible as only whole platelets, or TCA precipitates of whole platelets were analysed. It seemed, therefore, that if the inhibition of PG synthetase by aspirin depended upon the drug's ability to acetylate the enzyme, it should be possible to demonstrate the incorporation of labelled acetyl groups into the microsomal preparations of the enzyme described previously in this work. At about this time Roth and Majerus (1975) reported that incubation of human

platelets with /¯acetyl-^{3}H¯/ aspirin produced a rapid, saturable, irreversible acetylation of a platelet particulate fraction protein whose molecular weight was estimated at 85,000. The correlation between these findings and previous physiological observations of the effect of aspirin on platelets led the authors to conclude that aspirin may exert its anti-platelet effect by acetylating a residue within an active site of the cyclo-oxygenase component of PG synthetase.

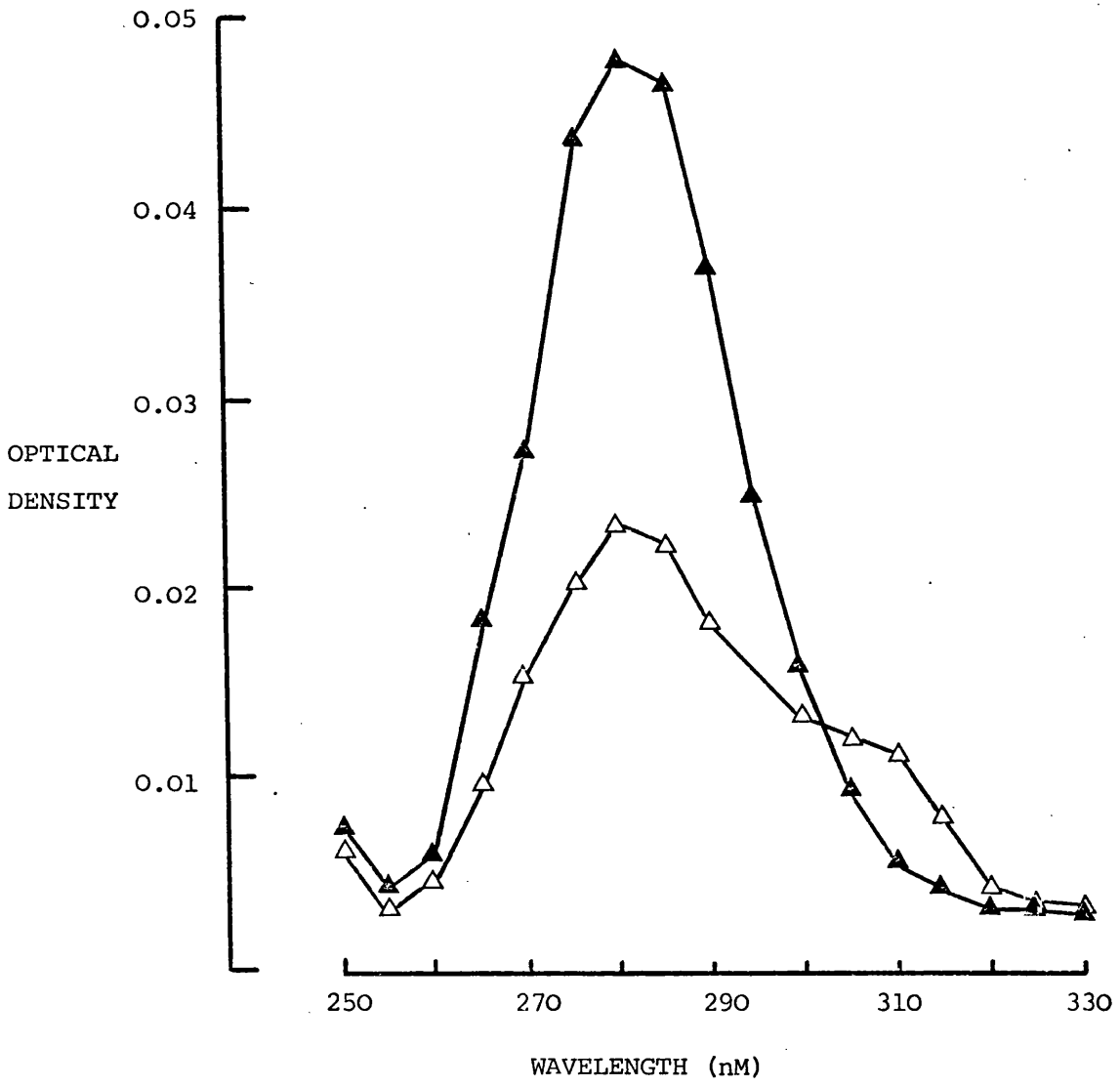
It was decided, therefore, to synthesise /¯acetyl-^{3}H¯/ aspirin of high specific activity, firstly to confirm the findings described above, in view of their relevance to the results obtained in the present work, and secondly to extend the results by studying the interaction between /¯acetyl-^{3}H¯/ aspirin and R.A. synovial PG synthetase.

6.2 Methods

Preparation of /¯acetyl-^{3}H¯/ aspirin

Salicylic acid (2.5 mg) was dissolved in pyridine (2.0 ml, redistilled) and treated with ^{3}H-acetic anhydride (25 mCi, specific activity 3500 mCi/mmol., Radiochemical Centre, Amersham) at 37^{o}c for 3 hr. The solvent was evaporated under nitrogen and the product purified by thin-layer chromatography (silica gel G, 0.25mm, methanol washed; solvent system cyclohexane-chloroform-acetic acid, 80:20:10). After radioscanning, the band corresponding to authentic aspirin, run in parallel, was transferred to a small sinter, eluted with redistilled ethanol (10 ml) and stored at -20^{o}c. Chromatographic properties and absorption spectra of the purified product showed it to be identical to authentic aspirin (Fig 6.1). Yield of /¯acetyl-^{3}H¯/ aspirin 10.8mCi (86% of theoretical).

Fig. 6.1 Absorption spectrum of synthesised /¯acetyl-^{3}H_/¯ aspirin



/¯acetyl-^{3}H_/¯ aspirin was synthesised as described in section 6.2 and its absorption spectrum measured in absolute ethanol (108μCi, 11.0μg, in 1.0ml). The two curves show the spectrum with a fresh preparation (▲——▲), and after 3 months storage in ethanol at -20°c (△——△).

Incubation of PG synthetase with /¯acetyl-^{3}H¯/ aspirin

Platelet or synovial microsomes, prepared as described previously (section 2.3), were incubated with /¯acetyl-^{3}H¯/ aspirin (60μM) in 0.1M tris-acetate buffer, pH 7.4, at 37^{o} for 30 min. The suspension was then layered onto tris-acetate buffer (0.1M, pH 7.4) containing sucrose (0.25M) and a pellet obtained by centrifugation at 10^{5}g for 1 hr.

Polyacrylamide gel electrophoresis of acetylated PG synthetase

To the pellet obtained as described above was added 200μl of 0.1M phosphate buffer, pH 7.1, containing 0.2% sodium dodecyl sulphate (S.D.S.), and 50μl of a solution of 20% S.D.S. and 0.5M mercaptoethanol in 0.1M phosphate buffer, pH 7.1. The mixture was boiled for 10 mins., and aliquots (50-100μl) run on 5% polyacrylamide gels in 0.1M phosphate buffer, pH 7.1 essentially as described by Weber and Osborn (1969). Electrophoresis was carried out using a constant current of 7 mA/gel, giving a running time for a 9cm gel of about 6 hr. Gels were run until a marker dye (10μl of a 0.05% solution of bromophenol blue) had reached the bottom of the gel. The gels were sliced into 3 mm sections, digested by incubating for 48hr. at 56^{o}C with hydrogen peroxide solution (20vol, 0.8 ml), liquid scintillant added ('Unisolve') and the samples counted for radioactivity. Quench corrections were made using an external standard ratio method, the efficiencies being in the range 26-31%. Gels run in parallel, containing either microsomal preparations or protein markers of known molecular weight, were stained with Coomassie brilliant blue dye.

6.3 Results

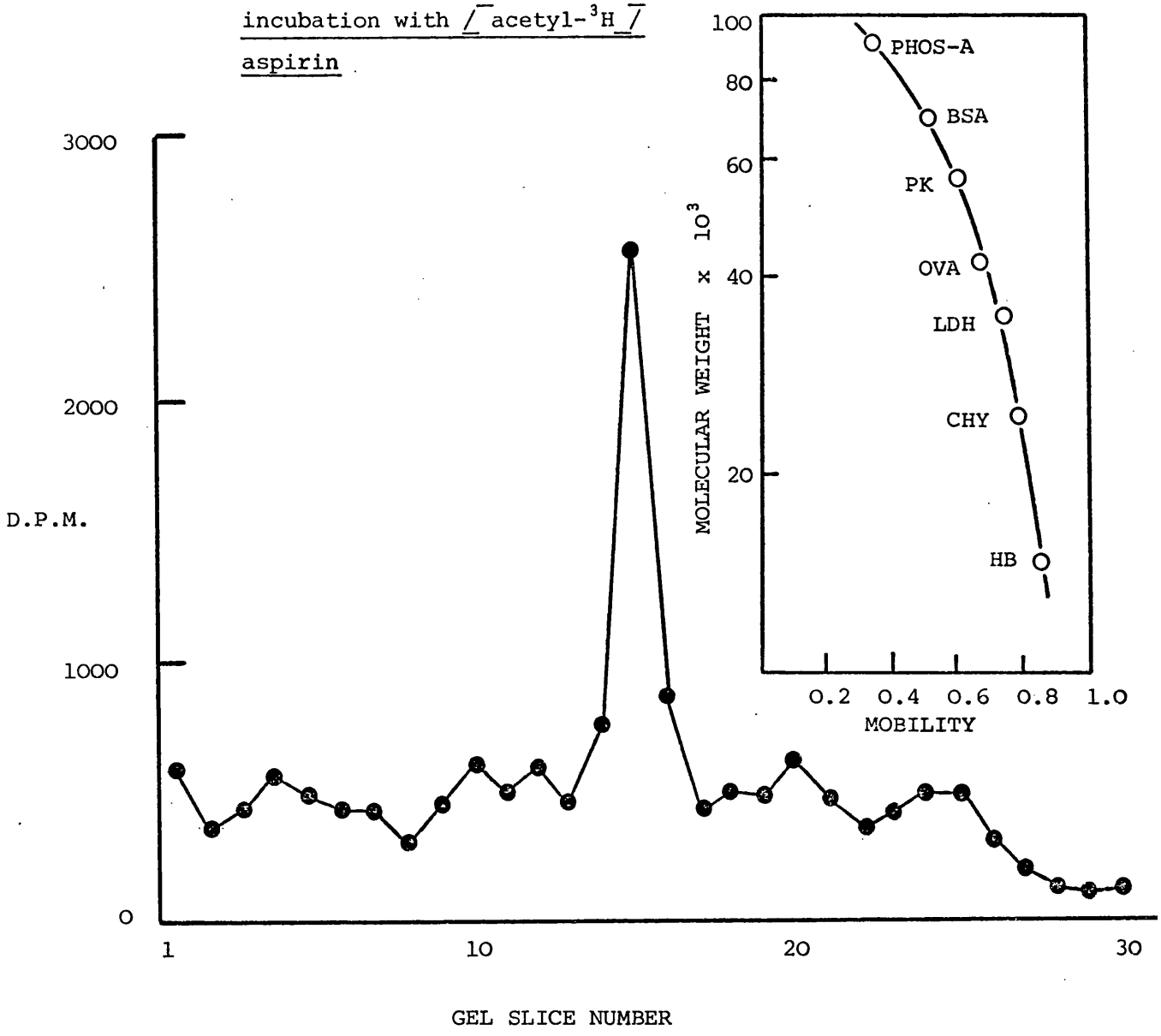
When human platelet microsomes were incubated with /¯acetyl-^{3}H¯/

aspirin of high specific activity, and the solubilised products subjected to polyacrylamide gel electrophoresis, a single peak of radioactivity labelled protein was seen (Fig 6.2). By reference to the calibration curve obtained from a series of proteins of known molecular weight (shown in the inset) the molecular weight of this labelled protein was found to be 81,000 in agreement with the figure reported by Roth and Majerus (1975) of 85,000. Calculation of the amount of label incorporated into the protein gave a figure of 2,000-3,000 acetyl groups per platelet, also in agreement with the above report. Calculation also indicates that the microsomal preparation used is, as might be expected, a highly impure form of PG synthetase; on the assumption that aspirin and the 85,000 molecular weight protein react on an equimolar basis, the microsomal preparation contains only 0.05% of this protein. Even if one hundred acetyl groups attach to one protein molecule the preparation only contains 5% of the protein, based on the total protein content of the microsomes.

When attempts were made to repeat the above experiment, but using R.A. synovial microsomes, much less satisfactory results were obtained. As with the platelet microsomes a labelled peak with a molecular weight of 81,000 was found (Fig. 6.3); however the amount of radioactivity incorporated into the peak was much less than was found in the platelet preparation; if the labelled peak is the cyclo-oxygenase component of PG synthetase (or at least a sub-unit of it) then this result is in accord with the findings in previous chapters that the level of PG synthetase is much higher in platelet microsomes than in R.A. synovial microsomes.

A further difference between the two preparations was seen when the polyacrylamide gels were stained with Coomassie brilliant blue; whereas the platelet preparation gave numerous clearly defined

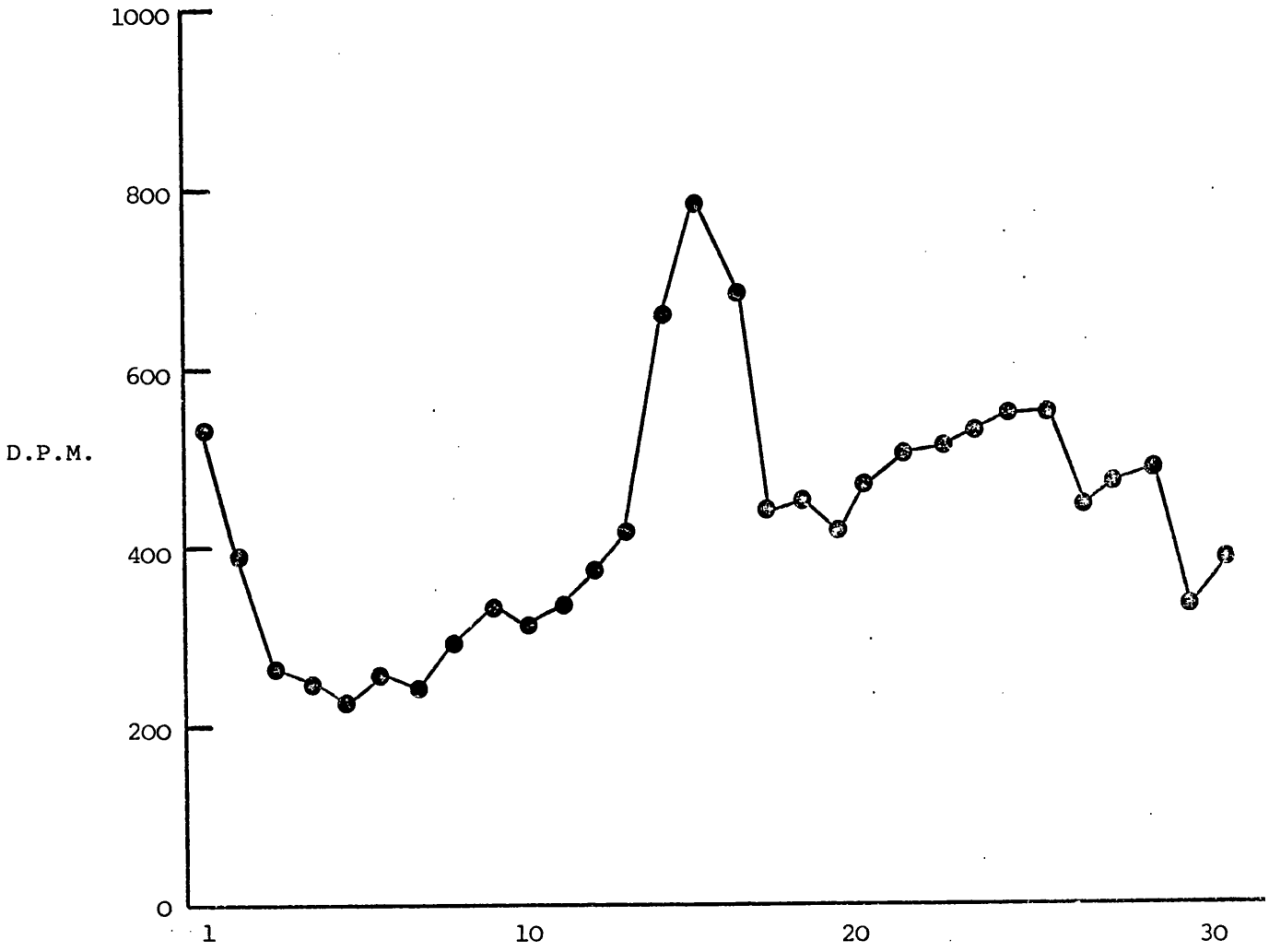
Fig. 6.2 Polyacrylamide/S.D.S. gel electrophoresis of solubilised human platelet microsomes after incubation with /¯acetyl-^{3}H¯/ aspirin



Human platelet microsomes were incubated with /¯acetyl-^{3}H¯/ aspirin (60μM) in 0.1 M tris-acetate buffer, pH 7.4, at 37°c for 30 min. The washed microsomes were solubilised with S.D.S./mercaptethanol and an aliquot containing 170μg protein electrophoresed on 5% polyacrylamide gel. The radioactivity in each gel slice was measured by liquid scintillation counting and the peak of activity calculated to have a molecular weight of 81,000.

Standard protein markers used were: BSA, bovine serum albumin; PHOS-A, phosphorylase-A; PK, pyruvate kinase; OVA, ovalbumin; LDH, lactic dehydrogenase; CHY, chymotrypsinogen; HB, haemoglobin.

Fig. 6.3 Polyacrylamide /S.D.S. gel electrophoresis of solubilised R.A. synovial microsomes after incubation with /¯acetyl-^{3}H¯/ aspirin.



R.A. synovial microsomes were incubated with /¯acetyl-^{3}H¯/ aspirin (60μM) in 0.1M tris-acetate buffer, pH 7.4, at 37°c for 30 min. The washed microsomes were solubilised with S.D.S./mercaptoethanol and an aliquot containing 310μg protein electrophorsed on 5% polyacrylamide gel. The radioactivity in each gel slice was measured by liquid scintillation counting and the peak of activity found to have a molecular weight of 81,000.

Note the enlarged scale on the ordinate compared with Fig. 6.2.

bands on staining, the synovial preparation produced a diffuse stain throughout the length of the gel and no bands were seen at all. The possibility that the gels containing solubilised synovial microsomes were either overloaded or streaking, due to the rather impure nature of the synovial preparation, was investigated by adding several protein markers of known molecular weight to synovial preparations and subjecting to gel electrophoresis followed by staining. The finding that the protein markers ran in the correct positions as sharp bands suggested that the synovial preparation was in fact suitable for gel electrophoresis. Attempts to produce a lipid-free preparation of acetylated microsomes, using either petroleum ether or acetone/pentane mixtures, followed by gel electrophoresis, were uniformly unsuccessful.

Studies had originally been planned to investigate the possible interaction of other aspirin-like drugs with the aspirin-microsome system; the finding that the potencies of the aspirin-like drugs as competitive inhibitors of the acetylation reaction correlated well with their potencies as inhibitors of PG synthetase would have provided good (albeit circumstancial) evidence that the labelled peak was indeed the cyclo-oxygenase component of the enzyme. An initial attempt was made to study this possible effect using indomethacin but no results were obtained, both due to the inconsistency of control incubations and also the very low levels of activity present once the control acetylation was inhibited. As the radioactively labelled aspirin used was synthesised from the highest specific activity reagent commercially available it seems unlikely that the technique described will yield good results, unless the enzyme preparation being studied contains a high level of PG synthetase activity.

Consideration was given to the possibility of subjecting cells labelled with /¯acetyl-^{3}H_/¯ aspirin to autoradiography in order

to locate the site of the PG synthetase within the cell. However, calculation showed that even in the platelet, which contains a high level of the enzyme, on the basis of 2000-3000 acetyl groups incorporated per platelet, autoradiography of a single labelled cell would yield approximately one disintegration per day due to ^{3}H-acetyl groups. A further complication would be that proteins within the cytoplasm may become labelled with ^{3}H-acetyl groups (Roth and Majerus, 1975).

The results obtained using /¯acetyl-^{3}H¯/ aspirin were therefore disappointing and suggest that although the use of labelled aspirin as a tool for detecting sites of PG synthesis is extremely attractive, the practicalities of such experiments may be fraught with difficulties.

6.4 Discussion

The use of /¯acetyl-^{3}H¯/ aspirin has provided the first results of the way in which aspirin interacts with PG synthetase at the molecular level. The initial report of Roth and Majerus (1975) showed that aspirin acted as an acetylating agent, as might have been suspected from previous work; acetyl-labelled aspirin produced incorporation of activity into a particulate fraction protein whereas ring-labelled aspirin did not. A later paper (Roth, Stanford and Majerus, 1975) showed that this interaction between aspirin and PG synthetase was not confined to platelets but also occurred with the enzyme prepared from ovine or bovine seminal vesicles. The finding that all three preparations of the enzyme gave a labelled peak of molecular weight 85,000 suggests that the technique is unlikely to reveal isoenzymes of significantly different molecular weights. The results obtained in the present work fully support the findings described above; R.A. synovial microsomal PG synthetase produced a labelled peak with an estimated molecular weight of 81,000. Future studies will probably show that the phenomenon is a general one,

though it would clearly be of interest to find a preparation of PG synthetase which did not interact with aspirin in this way.

The very low levels of radioactivity incorporated into the enzyme in the present study, and in the previous reports described, suggests that lack of sensitivity may be a limiting factor in the use of /¯acetyl-^{3}H¯/ aspirin as a probe for detecting sites of PG synthesis. Seminal vesicular glands and platelets are known to contain high levels of PG synthetase and R.A. synovial tissue contains the same order of magnitude of activity as rabbit renal medulla, known to be a good source of the enzyme (Christ and van Dorp, 1972). It may be extremely difficult therefore to demonstrate the incorporation of radioactivity into preparations containing low levels of the enzyme.

The finding that the radioactively-labelled protein peak seen in the present work has a molecular weight of 81,000 is of interest in the light of the report of Miyamoto and others (1976). These authors showed that a purified 'prostaglandin endoperoxide synthetase', i.e. cyclo-oxygenase, prepared from bovine vesicular glands, has a molecular weight of 300,000-350,000, estimated using gel filtration on Sephadex G-200. It is tempting to speculate that the labelled peak found on gel electrophoresis may be a quarter subunit of the cyclo-oxygenase component of PG synthetase.

CHAPTER SEVEN

GENERAL DISCUSSION

The work presented in this thesis centres around the proposal put forward by Vane in 1971 that the aspirin-like drugs exert their pharmacological properties by inhibiting the production of prostaglandins. A development of the original proposal was that PG synthetase exists within an organism in multiple molecular forms, each isoenzyme possessing a characteristic pharmacological 'profile' with respect to inhibition by the aspirin-like drugs (Vane, 1972). As most studies of PG synthetase have been carried out on the enzyme prepared from highly active animal tissues the relevance of studying the interaction between the aspirin-like drugs used in clinical practice and the enzyme prepared from human inflammatory tissue is self-evident.

Evidence for the existence of PG synthetase isoenzymes hinges largely upon the report of Flower and Vane (1972) that paracetamol is a much more potent inhibitor of brain PG synthetase than of the enzyme prepared from peripheral tissues such as spleen. The findings with this one, possibly atypical, drug have led to speculation that an ideal aspirin-like drug might be developed. Such a drug would be a potent inhibitor of the PG synthetase of inflammatory tissue, yet virtually inactive as an inhibitor of the enzyme found in other organs such as stomach or kidney, in which aspirin-like drugs may produce adverse side-effects via inhibition of PG synthesis.

The present author is highly sceptical that such a drug may be developed, for a recent report (Pong and Levine, 1976) has shown the PG synthetase prepared from seven different rabbit tissues to be inhibited identically by indomethacin, aspirin or flufenamic acid. Unfortunately these authors did not examine the potency of paracetamol as an inhibitor of the various preparations. If paracetamol is inactive as an inhibitor of PG synthetase in peripheral tissues, (as found in the present work), it is difficult to explain the drug's analgesic

activity in terms of inhibition of PG synthesis, though its lack of anti-inflammatory activity may be explained on this basis.

During the course of the present study no evidence was found that PG synthetase prepared from human inflammatory tissue differed radically, either biochemically or pharmacologically, from the well-studied systems prepared from animal tissues. It is possible that such differences as have been reported (Blackwell, Flower and Vane, 1975) may be a reflection of the relatively crude enzyme preparations employed and incubation conditions *in vitro* differing drastically from those existing *in vivo*.

A finding of great interest in the present work was that PG synthetase activity was abolished in the microsomal preparations from the synovial tissue of three patients receiving only 'analgesic' (600 mg/day) doses of aspirin. If such a dose is able to inactivate the enzyme in the target tissue, and if aspirin exerts its anti-inflammatory properties through inhibition of PG synthetase, it is relevant to ask why much larger doses are necessary to demonstrate anti-inflammatory activity in clinical practice. This argument has been expounded in detail by others (Smith, 1975; Smith, Ford-Hutchinson and Elliott, 1975); perhaps further evidence of this nature may help to displace prostaglandins from their current 'monopolistic' position in order that other facets of the mode of action of aspirin-like drugs may be objectively investigated.

In this respect the recent report of Bonta and others (1977) is of interest. These authors report that both aspirin and dexamethasone are effective in suppressing carrageenin-induced oedema in both normal and essential fatty acid-deficient (EFAD) rats, even though, in the latter, the role of an activated PG system can be ruled

out due to lack of endogenous PG precursors. The results suggest that the anti-inflammatory effect of these drugs involves at least one mechanism which is independent of PG synthesis inhibition, possibilities including inhibition of leucocyte migration, inhibition of phosphodiesterase and formation of copper complexes. The EFAD rat may thus be a useful tool for investigating mechanisms which in normal animals may be masked by interference with PG production. The present author is convinced that future work will show the aspirin-like drugs to have multiple interactions in the inflammatory process (Whitehouse, 1968).

Theories are not overthrown by criticism, but by better theories, and it will be of interest to follow the development of new anti-inflammatory drugs which are not inhibitors of PG synthetase. Reports of anti-inflammatory peptides prepared from either bee venom (Billingham and others, 1973) or human plasma (Smith and others, 1974) have shown that these compounds do not interfere with the PG system, but may act by preventing the release of leucotactic factors when the complement cascade is activated by the alternate, i.e. non-immune, pathway.

One of the most interesting reports to appear recently is that of Boyle and others (1976). These authors find that whilst the copper complexes of clopirac, niflumic acid and aspirin possess the same anti-inflammatory potency as their parent compounds, as judged by suppression of carrageenin-induced oedema in the rat paw, they are considerably less ulcerogenic. This in itself is interesting, but the surprising finding is that these copper complexes are much less potent inhibitors of PG synthetase <u>in vitro</u> than their parent compounds. Just why this is the case, and why indomethacin, ketoprofen and naproxen do not behave in this way are questions which remain to be answered.

It would indeed be ironical if the inhibition of PG synthetase in vitro were to become a screening test for ulcerogenicity rather than anti-inflammatory activity! That this is not merely academic speculation is shown by the finding that a new anti-inflammatory drug (LRCL 3794) currently under investigation has been found to be a very weak inhibitor of PG synthetase in vitro (W. Dawson, personal communication). Similarly, the drug designated Abbott-29590 has been reported to be inactive as a PG synthetase inhibitor in vitro and to produce no gastric lesions in fasted rats at doses up to 526 mg/kg. (Carter and others, 1974). A low incidence of gastro-intestinal side effects produced by these compounds at the clinical trial stage would indeed be a finding of the greatest significance, and may herald a new class of aspirin-like drugs whose toxicity is (hopefully) far from aspirin-like. Perhaps future antirheumatic drugs will not be subject to the jibe concerning present conventional medications that they are "chemically a ragbag and therapeutically rather a disaster area".

A future challenge for the pharmacologist will be the development of a screening test to detect the kind of antirheumatic activity shown by drugs such as penicillamine, chloroquine and gold salts. Such compounds, with a slow onset of action, have in the past been discovered by chance and their mode of action is poorly understood. Perhaps in such compounds lies the possibility of a drug which may actually slow or halt the disease process, though the formidable toxicity of the drugs mentioned above suggests that it may be difficult to divorce their antirheumatic properties from their unwanted side-effects. The same argument may hold true for the development of future immunosuppressive drugs.

When the potencies of the commonly used aspirin-like drugs as inhibitors of R.A. synovial PG synthetase in vitro were compared with

their known therapeutic potencies *in vivo*, the *in vitro* measurement was found to give a much greater potency, relative to aspirin, than is known for therapeutic activity. Whether this is an indication that inhibition of PG synthesis is not the sole, or even the most important factor governing therapeutic potency remains to be seen. Certainly one must interpret results obtained from *in vitro* studies with caution, as a drug studied *in vitro* does not have to overcome problems of absorption, distribution, metabolism and excretion before reaching its side of action, as is the case *in vivo*. Conversely some drugs may acquire anti-inflammatory activity only after metabolism *in vivo*, though as discussed previously the case for salicylic acid behaving in this way is not convincing.

Until more information is available concerning the precise working of the enzyme *in vivo*, such as the nature of cofactors, their concentrations and availability to the enzyme, substrate concentration(s) and availability, and the presence of 'natural' inhibitors, one must concede that an *in vitro* system is somewhat artificial and results obtained from it may only be extrapolated to the situation *in vivo* with caution.

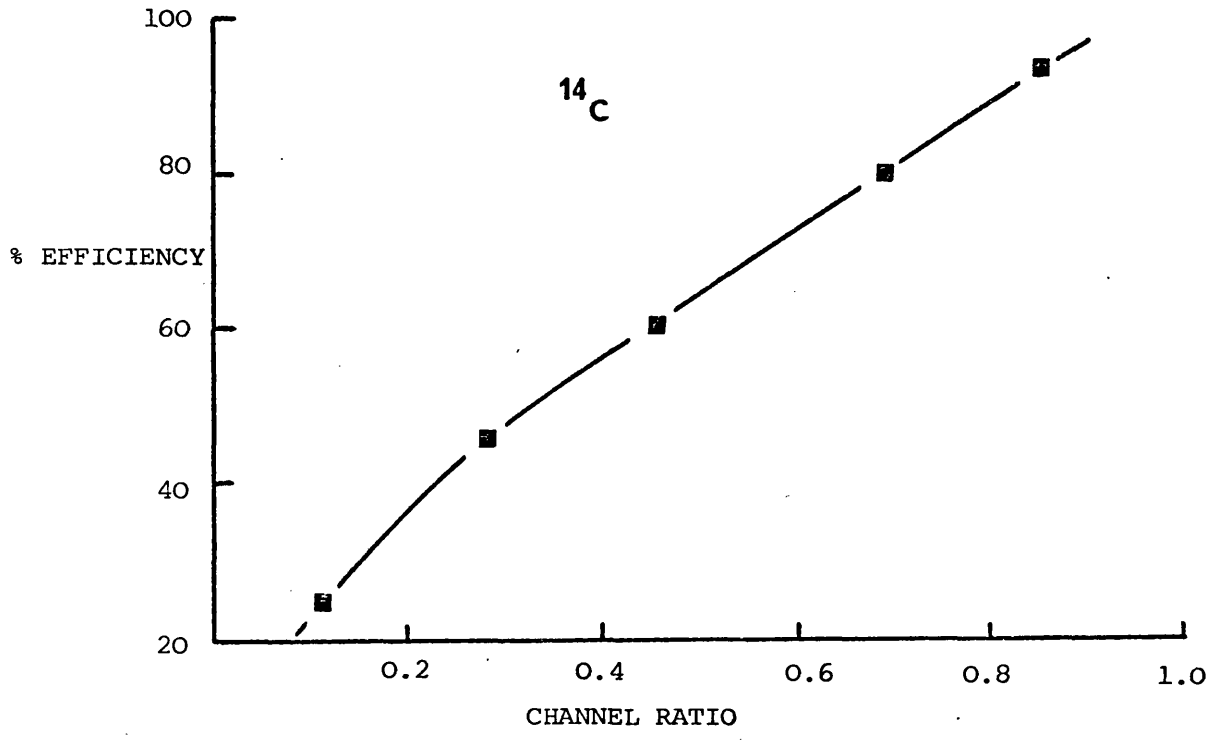
An interesting finding to emerge from the present work, at least from a biochemical point of view, is that aspirin is an irreversible inhibitor of human synovial and platelet PG synthetase, both *in vitro* and *in vivo*, and that aspirin may be unique in this respect. One might therefore expect the time course of PG synthetase inhibition *in vivo* to be more prolonged with aspirin than one might predict from its relatively short half-life in the blood, and this is certainly true for platelets where no *de novo* enzyme synthesis is possible. The situation in tissue such as synovium however is much more complex as little is known of the turnover rates of PG synthetase in either synovial cells

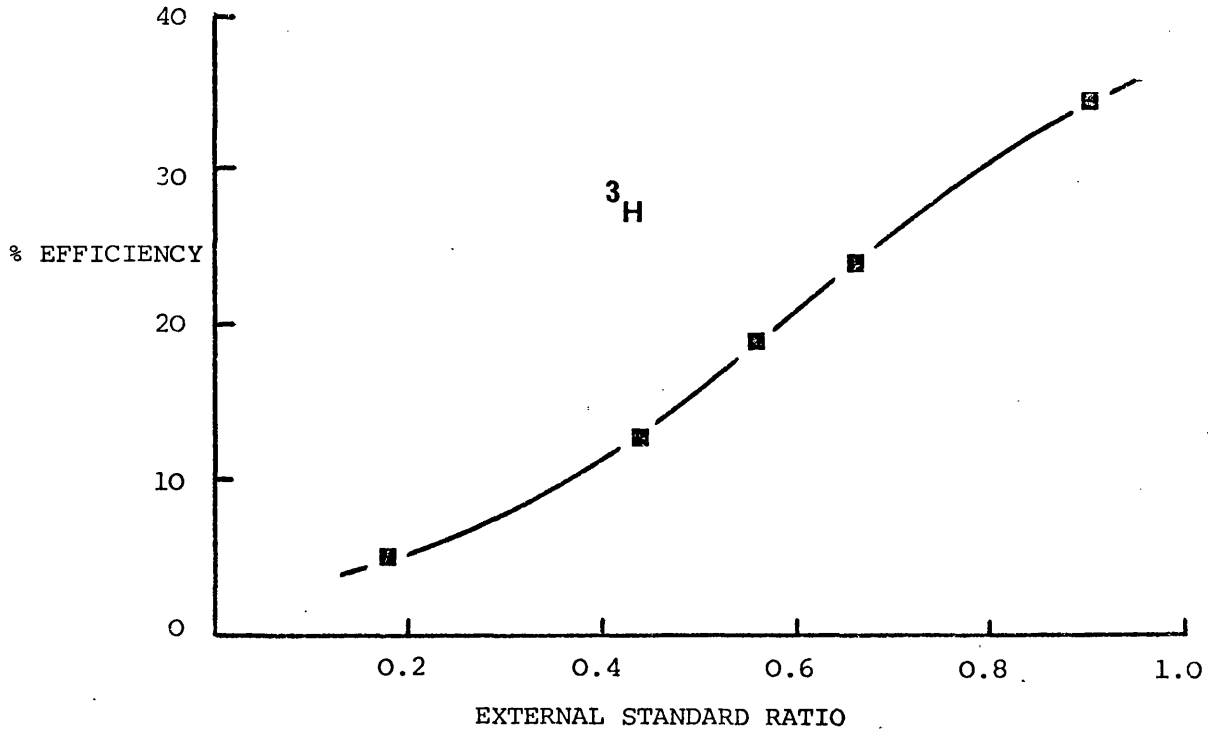
or invading leucocytes. If, for example, the turnover rate of the enzyme in synovial cells is high, then the action of an irreversible inhibitor of the enzyme *in vivo* may be difficult to distinguish from that of a reversible inhibitor.

APPENDIX

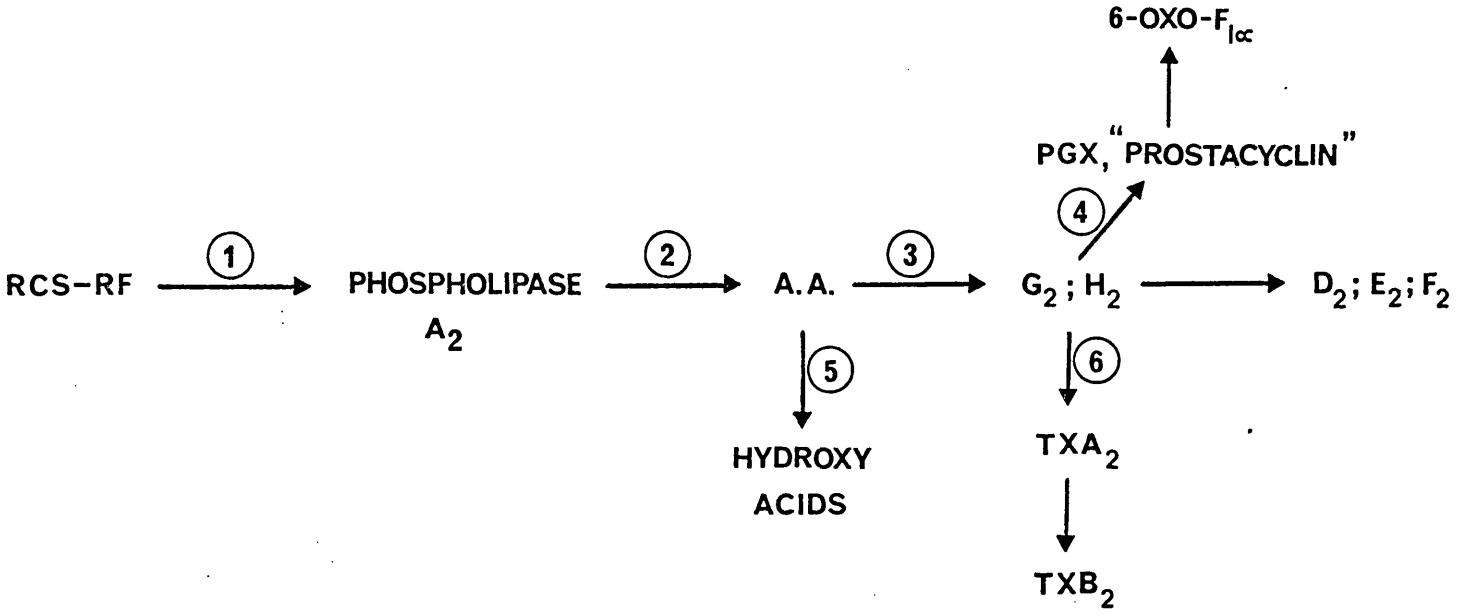
Appendix 1

Quench correction curves for liquid scintillation counting





Appendix 2 Points at which various inhibitors are thought to act.



1. ANTI-INFLAMMATORY STEROIDS
2. ANTI-MALARIALS
3. ASPIRIN-LIKE DRUGS
4. 15-HYDROPEROXY-ARACHIDONIC ACID
5. TYA
6. BENZYDAMINE

REFERENCES

Åberg, G. (1973). Int.Res.Comm.Syst. 73-4, 9-3-3. (Interactions between salicylates and prostaglandins on the temperature of inflamed rat paw).

Adams, S.S., Bresloff, P. and Mason, C.G. (1975). Scand. J. Rheum.suppl.8. Abstract S08-13 (The optical isomers of Ibuprofen).

Adams, S.S. and Cobb, R. (1958) Nature 181, 773-774. (A possible basis for the anti-inflammatory activity of salicylates and other non-hormonal anti-rheumatic drugs).

Adams, S.S. and Cobb, R. (1967). In: Progess in Medicinal Chemistry 5, p.59-138. Butterworths, London.

Ahern, D.G. and Downing, D.T. (1970). Biochim.Biophys.Acta. 210, 456-461. (Inhibition of prostaglandin biosynthesis by eicosa-5,8,11,14-tetraynoic acid).

Aizawa, Y. and Yamada, K. (1974). Jap.J.Pharmac. 24, 647-649. (Radioisotope dilution method for the determination of prostaglandin $F_{2\alpha}$ in rat tissue).

Al-Mondhiry, H., Marcus, A.J. and Spaet, T.H. (1970). Proc. Soc.Exp.Biol.Med. 133, 632-636. (On the mechanism of platelet function inhibition by acetylsalicylic acid).

Alpen, E.L., Mandell, H.G., Rodwell, V.W. and Smith, P.K. (1951). J.Pharmac.Exp.Ther. 102, 150-155. (The metabolism of C-14 carboxyl salicylic acid in the dog and in man).

Ambache, N. (1962). J.Physiol.(Lond.) 160, 3P. (Prolonged erythema produced by chromatographically purified "irin").

Andersen, N.H. and Leovey, E.M.K. (1974). Prostaglandins 6, 361-375. (Identification and quantitative determination of prostaglandins by high pressure liquid chromatography).

Andrieu, J.M., Mamas, S. and Dray, F. (1974). Prostaglandins 6, 15-23. (Viroimmunoassay of prostaglandin $F_{2\alpha}$ at the picogram level).

Änggård, E., Larsson, C. and Samuelsson, B. (1971). Acta. Physiol.Scand. 81, 396-404. (The distribution of 15-hydroxy-prostaglandin dehydrogenase and prostaglandin $-\Delta^{13}$ - reductase in tissues of the swine).

Änggård, E. and Samuelsson, B. (1964). J.biol.Chem. 239, 4097-4102. (Prostaglandins and related factors 28. Metabolism of prostaglandin E_1 in guinea pig lung: the structures of two metabolites).

Änggård, E. and Samuelsson, B. (1966). Ark. Kemi. 25, 293-300. (Purification and properties of a 15-hydroxy-prostaglandin dehydrogenase from swine lung).

Arora, S., Lahiri, P.K. and Sanyal, R.K. (1970). Int. Arch. Allergy. Appl. Immunol. 39, 186-191 (The role of prostaglandin E, in inflammatory process in the rat).

Bailey, E. and West, H.F. (1967). Lancet ii, 1231-1232. Prednisolone metabolism and rheumatoid arthritis).

Barland, P. (1973). Amer. J. Med. 54, 143-147. (Possible relationships between an infectious agent and the immune reactions of rheumatoid synovitis).

Barnado, D.E., Currey, H.L.F., Mason, R.M., Fox, W.R. and Weatherall, M. (1966). Brit. Med. J. ii, 342-343. (Mefenamic acid and flufenamic acid compared with asprin and phenylbutazone in rheumatoid arthritis).

Baumagartner, H.R. (1974). In: Thrombosis-Pathogenesis and Clinical Trials. Ed. Deutch, E. p.91. Schattauer, Stuttgart and New York. (The subendothelial surface and thrombosis).

Bergström, S., Danielsson, H., Klenberg, D. and Samuelsson, B. (1964). J. biol. Chem. 239, pc.4006-pc.4008. (The enzymic conversion of essential fatty acids into prostaglandins).

Bergström, S., Danielsson, H. and Samuelsson, B. (1964). Biochim. Biophys. Acta. 90, 207-210. (The enzymatic formation of prostaglandin E_2 from arachidonic acid. Prostaglandins and related factors 32).

Bergström, S., Duner, H., von Euler, U.S., Pernow, B. and Sjovall, J. (1959). Acta. Physiol. Scand. 45, 145-151. (Observations on the effects of infusions of prostaglandin E in man).

Bhattacherjee, P. and Eakins, K.E. (1974). Brit. J. Pharmac. 50, 227-230. (Inhibition of the prostaglandin synthetase system in ocular tissues by indomethacin).

Bjørnstad, P. (1966). Biochim. Biophys. Acta. 116, 500-510. (Phospholipase activity in rat-liver microsomes studied by the use of endogenous substrates).

Blackham, A., Farmer, J.N., Radziwonik, H. and Westwick, J. (1973). Brit. J. Pharmac. 48, 343P-344P. (Rabbit monoarticular arthritis and synovial prostaglandins).

Blackwell, G.J., Flower, R.J. and Vane, J.R. (1975). Biochim. Biophys. Acta. 398, 178-190. (Some characteristics of the prostaglandin synthesising system in rabbit kidney microsomes).

Blumenkrantz, N. and Søndergaard, J. (1972). Nature (New Biol.) 239, 246-247. (Effect of prostaglandins E_1 and $F_{1\alpha}$ on biosynthesis of collagen).

Boardman,P.L. and Hart,F.D. (1967). Brit.Med.J. 4, 264-268. (Clinical measurement of the anti-inflammatory effects of salicylate in rheumatoid arthritis).

Bohman,S.-O. and Larsson,C. (1975). Acta.Physiol.Scand. 94, 244-258 (Prostaglandin synthesis in membrane fractions from the rabbit renal medulla).

Bonta,I.L., Bult,H., Vincent,J.E. and Zijlstra,F.J. (1977). J.Pharm.Pharmac. 29, 1-7 (Acute anti-inflammatory effects of asprin and dexamethasone in rats deprived of endogenous prostaglandin precursors).

Bourne,H.R., Lichtenstein,L.M. and Melmon,K.L. (1972). J.Immunol. 108, 695-705 (Pharmacologic control of allergic histamine release in vitro: Evidence for an inhibitory role of 3'5'-adenosine monophosphate in leucocytes).

Boyum,A. (1968). Scand.J.Clin.Lab.Invest. 21, Suppl. 97, 9-76. (Isolation of leucocytes from human blood).

Brocklehurst,W.E. and Dawson,W. (1974). In: Future Trends in Inflammation. Eds. Velo,G.P., Willoughby,D.A. and Giroud,J.P. p.37-44. Piccin Medical Books, Padua. (New data concerning the inhibition of prostaglandin formation by anti-inflammatory drugs).

Bywaters,E.G.L. (1963). In: Salicylates,An International Symposium. p.64. Churchill, London.

Carter,G.W., Dodge,D.W., Krause,R.A., Young,P.R. and Sweet,L.R. (1974). Fed.Proc. 33, 557. (The apparent lack of PG synthetase inhibition by Abbott-29590: 2,3-dihydro-1-H-pyridino (2,3-)(1,4) - thiazine-2-one, an anti-inflammatory agent).

Chan,J.A., Nagasawa,M., Takeguchi,C. and Sih,C.J. (1975). Biochemistry 14, 2987-2991 (On agents favoring prostaglandin F formation during biosynthesis).

Chang,Y-H. (1972). J.Pharmac.Exp.Ther. 183, 235-244. (Studies on phagocytosis. II. The effect of nonsteroidal anti-inflammatory drugs on phagocytosis and on urate crystal-induced canine joint inflammation).

Christ,E.J., Van Dorp,D.A. (1972). Biochim.Biophys.Acta. 270, 537-545. (Comparative aspects of prostaglandin biosynthesis in animal tissues).

Cochrane,C.G. (1968). J.Allergy. 42, 113-121. (The role of immune complexes and complement in tissue injury).

Collier,H.O.J. (1969). Adv.Pharmac.Chemother. 7, 333-405. (A pharmacological analysis of aspirin).

Cushman, D.W. and Cheung, H.S. (1976). Biochim. Biophys. Acta. 424, 449-459. (Effect of substrate concentration on inhibition of prostaglandin synthetase of bull seminal vesicles by anti-inflammatory drugs and fenamic acid analogs).

Dayer, J-M., Krane, S.M., Russell, G.G. and Robinson, D.R. (1976). Proc. Natl. Acad. Sci. U.S.A. 73, 945-949. (Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells).

Deamer, D.W., Heikkila, R.E., Panganamala, R.V., Cohen, G. and Cornwell, D.G. (1971). Physiol. Chem. Phys. 3, 426-433. (The alloxan-dialuric acid cycle and the generation of hydrogen peroxide).

Deby, C., Bacq, Z.M. and Simon, D. (1973). Biochem. Pharmac. 22, 3141-3143. (Gold and silver inhibition of prostaglandin synthetase).

DeDuve, C. (1971). J. Cell. Biol. 50, 20d-55d. Tissue fractionation past and present).

DeFuria, F.G., Bridges, K.F., Bunn, H.F., Lee, Y.S., Peterson, C.M., Schmidt, G., Jensen, M. and Cerami, A. (1974). 1st International Symposium on Sickle Cell Disease. 185-186. (The acetylation of hemoglobin by aspirin).

DeHaas, G.H., Postema, N.M., Nievwenhuizen, W. and vanDeenen, L.L.M. (1968). Biochim. Biophys. Acta. 159, 103-117. (Purification and properties of phospholipase A from porcine pancreas).

Destephano, D.B., Brady, U.E. and Woodall, L.B. (1976). Prostaglandins 11, 261-273. (Partial characterisation of prostaglandin synthetase in the reproductive tract of the male house cricket, acheta domesticus).

Dioguardi, N., Agostoni, A., Fiorelli, G. and Lomanto, B. (1963). J. Lab. Clin. Med. 61, 713-723. (Characterisation of lactic dehydrogenase of normal human granulocytes).

Dipasquale, G., Rassaert, C., Richter, R., Welaj, P. and Tripp, L. (1973). Prostaglandins 3, 741-757. (The influence of PGE_2 and PGF_2 on the inflammatory process).

DiRosa, M., Giroud, J.P. and Willoughby, D.A. (1971). J. Pathol. Bacteriol. 104, 15-29. (Studies of the mediators of the acute inflammatory response induced in rats in different sites by carrageenin and turpentine).

Dresner, E. and Trombly, P. (1960). Clin. Res. 8, 16. (Chemical dissociation of the rheumatoid factor in vitro and in vivo).

Eakins, K.E., Whitelocke, R.I.F., Bennett, A. and Martenet, A.C. (1972). Brit. Med. J. 3, 452-453. (Prostaglandin-like activity in ocular inflammation).

Eakins, K.E., Whitelocke, R.A.F., Perkins, E.S., Bennett, A. and Unger, W.G. (1972) Nature (New Biol.) 239, 248-249. (Release of prostaglandin in ocular inflammation in the rabbit).

Ebert, R.H. and Grant, L. (1974). In: The Inflammatory Process Eds. Zweifach, B.W., Grant, L. and McCluskey, R.T. p.4. Academic Press, New York, San Francisco and London. (The Experimental approach to the study of inflammation).

Egan, R.W. (1975). Anal. Biochem. 68, 654-657. (A general method for quantitative separation of prostaglandins by paper chromatography).

Eisenbarth, G.S., Beuttel, S.C. and Lebovitz, H.E. (1974). J. Pharmac. Exp. Ther. 189, 213-220. (Inhibition of cartilage macromolecular synthesis by prostaglandin A_1).

Eisenthal, R. and Cornish-Bowden, A. (1974). Biochem. J. 139, 715-720. (The direct linear plot).

Elmes, M.E. (1974). Lancet, 1329-1330 (Anti-inflammatory drugs and tissue copper).

English, J., Chakraborty, J. and Marks, V. (1974). Ann. Clin. Biochem. 11, 11-14. (A competitive protein binding method for plasma prednisolone assay).

Evanson, J.M., Jeffrey, J.J. and Krane, S.M. (1968). J. Clin. Invest. 47, 2639-2651. (Studies on collagenase from rheumatoid arthritis synovium in tissue culture).

Famaey, J-P., Brooks, P.M. and Dick, W.C. (1975). Sem. Arth. Rheum. 5, 63-81. (Biological effects of nonsteroidal anti-inflammatory drugs).

Feldberg, W. and Gupta, K.P. (1973). J. Physiol. 228, 41-53. (Pyrogen fever and prostaglandin-like activity in cerebrospinal fluid).

Feldberg, W. and Saxena, P.N. (1971). J. Physiol. 217, 546-556. (Fever produced by prostaglandin E_1).

Ferraris, V.A. and DeRubertis, F.R. (1974). J. Clin. Invest. 54, 378-386. (Release of prostaglandin by mitogen- and antigen-stimulated leukocytes in culture).

Ferreira, S.H. (1972). Nature (New Biol.) 240, 200-203. (Prostaglandins, aspirin-like drugs and analgesia).

Ferreira, S.H. (1976). In: The Role of Prostaglandins in Inflammation. Ed. Lewis, G.P. p. 45. Hans Huber, Bern, Stuttgart and Vienna.

Ferreira, S.H., Moncada, S. and Vane, J.R. (1971). Nature (New Biol.) 231, 237-239. (Indomethacin and aspirin abolish prostaglandin release from the spleen).

Ferreira, S.H., Moncada, S. and Vane, J.R. (1973). Brit.J. Pharmac. 47, 629P-630P. (Further experiments to establish that the analgesic action of aspirin-like drugs depends upon the inhibition of prostaglandin biosynthesis).

Ferreira, S.H. and Vane, J.R. (1967). Nature (Lond) 216, 868-873. (Prostaglandins: their disappearance from and release into the circulation).

Ferreira, S.H. and Vane, J.R. (1974). Ann.Rev.Pharmac. 14, 57-73. (New aspects of the mode of action of non-steroid anti-inflammatory drugs).

Flower, R.J. (1974). Pharmac.Rev. 26, 33-67. (Drugs which inhibit prostaglandin biosynthesis).

Flower, R.J. and Blackwell, G.J. (1976). Biochem.Pharmac. 25, 285-291. (The importance of phospholipase-A_2 in prostaglandin biosynthesis).

Flower, R.J., Cheung, H.S. and Cushman, D.W. (1973). Prostaglandins 4, 325-341. (Quantitative determination of prostaglandins and malondialdehyde formed by the arachidonate oxygenase system of bovine seminal vesicles).

Flower, R.J. Gryglewski, R., Herbaczynska-Cedro, K. and Vane, J.R. (1972). Nature (New Biol.) 238, 104-106. (Effects of anti-inflammatory drugs on prostaglandin biosynthesis).

Flower, R.J. and McClure, W.O. (1975). Anal.Biochem. 68, 436-447. (A simple isotope derivative assay for prostaglandins and prostaglandin metabolites).

Flower, R.J. and Vane, J.R. (1972). Nature 240, 410-411. (Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol - 4-acetamidophenol).

Flower, R.J. and Vane, J.R. (1974). In: Prostaglandin Synthetase Inhibitors. Eds. Robinson, H.J. and Vane, J.R. p.9-18. Raven Press, New York. (Some pharmacological and biochemical aspects of prostaglandin biosynthesis and its inhibition).

Ford-Hutchinson, A.W., Smith, M.J.H. and Walker, J.R. (1976). Brit.J.Pharmac. 56, 345P-346P. (Chemotactic activity of solutions of prostaglandin E_1).

Frölich, J.C., Sweetman, B.J., Carr, K., Hollifield, J.W. and Oates, J.A. (1975). Prostaglandins 10, 185-195. (Assessment of the levels of prostaglandin A_2 in human plasma by gas chromatography-mass spectrometry).

Frölich, J.C., Wilson, T.W., Smigel, M. and Oates, J.A. (1974). Biochim. Biophys. Acta. 348, 241-248. (A competitive protein binding assay specific for prostaglandin E).

Gallus, A.S. and Hirsh, J. (1976). Drugs 12, 41-68. (Antithrombotic drugs: 1. Practical therapeutics).

Girdwood, R.H. (1974). Brit. Med. J. 1, 501-504, (Death after taking medicaments).

Glatt, M., Peskar, B. and Brune, K. (1974). Experientia 30, 1257-1259. (Leucocytes and prostaglandins in acute inflammation).

Glenn, E.M. Bowman, B.J. and Rohloff, N.A. (1972) In: Prostaglandins in Cellular Biology. Eds. Ramwell, P.W. and Phariss, B.B. p.329-343. Plenum Press, New York and London. (Pro-inflammatory effects of certain prostaglandins).

Glenn, E.M. and Rohloff, N.A. (1972). Proc. Soc. Exp. Biol. Med. 139, 290-294. (Anti-arthritic and anti-inflammatory effects of certain prostaglandins).

Glynn, L.E. (1972). Ann. rheum. Dis. 31, 412-420. (Pathology, pathogenesis and aetiology of rheumatoid arthritis).

Goldberg, N.D., Haddox, M.K., Hartle, D.K. and Hadden, J.W. (1973). In: Pharmacology and the Future of Man. p.146. Karger, Basel.

Graham, H.T., Lowry, O.H., Wheelwright, F. and Lenz, M.A. (1955). Blood 10, 467-481. (Distribution of histamine among leukocytes and platelets).

Granström, E., Inger, U. and Samuelsson, B. (1965). J. biol. Chem. 240, 457-461. (Prostaglandins and related factors 29. The structure of a urinary metabolite of prostaglandin $F_{1\alpha}$ in the rat).

Granström, E. and Samuelsson, B. (1971). J. biol. Chem. 246, 5254-5263. (On the metabolism of prostaglandin $F_{2\alpha}$ in female subjects).

Greaves, M.W. and McDonald-Gibson, W.J. (1972). Brit. Med. J. 2, 83-84. (Inhibition of prostaglandin biosynthesis by corticosteroids).

Greaves, M.W. and Søndergaard, J. (1970). J. Invest. Dermatol. 54, 365-367. (Pharmacological agents released in ultraviolet inflammation studied by continuous skin perfusion).

Greaves, M.W., Søndergaard, J. and McDonald-Gibson, W. (1971). Brit. Med. J. 2, 258-260. (Recovery of prostaglandins in human cutaneous inflammation).

Green,K. and Samuelsson,B. (1964). J.Lipid.Res. 5, 117-120. (Prostaglandins and related factors: XIX. Thin layer chromatography of prostaglandins).

Cryglewski,R., Flower,R.J., Herbazynska-Cedro,K. and Vane,J.R. (1972). Proceedings of the Fifth International Congress of Pharmacology, San Francisco. p.90. (Inhibition of prostaglandin synthetase by anti-inflammatory drugs).

Guzman,F., Braun,C. and Lim,R.K.S. (1962). Arch.Int. Pharmac.Ther. 136, 353-384. Visceral pain and the pseudo-affective response to intra-arterial injections of bradykinin and other algesic agents).

Ham,E.A., Cirello,V.J., Zanetti,M., Shen,T.Y. and Kuehl,F.A. (1972). In: Prostaglandins in Cellular Biology. Eds. Ramwell,P.W. and Phariss,B.B. p.343-352. Plenum Press, New York and London. (Studies on the mode of action of non-steroidal anti-inflammatory agents).

Hamberg,M. (1969). Febs.Lett. 5, 127-130. (Biosynthesis of prostaglandins in the renal medulla of rabbit).

Hamberg,M. (1972). Biochem.Biophys.Res.Comm. 49, 720-726. (Inhibition of prostaglandin synthesis in man).

Hamberg,M. and Israelsson,U. (1970). J.biol.Chem. 245, 5107-5114. (Metabolism of prostaglandin E_2 in guinea pig liver. 1. Identification of seven metabolites).

Hamberg,M. and Samuelsson,B. (1966). J.biol.Chem. 241, 257-263. (Prostaglandins and related factors. 46. Prostaglandins in human seminal plasma).

Hamberg,M. and Samuelsson,B. (1967). J.biol.Chem. 242: 5336-5343. (On the mechanism of the biosynthesis of prostaglandins E_1 and $F_{1\alpha}$).

Hamberg,M. and Samuelsson,B. (1968). Federation of Europ. Biochem. Soc. Meeting, Prague, 1968.

Hamberg,M. and Samuelsson,B. (1969). J.Amer.Chem.Soc. 91, 2177-2178. (The structure of the major urinary metabolite of prostaglandin E_2 in man).

Hamberg,M. and Samuelsson,B. (1974a). Proc.Natl. Acad.Sci. U.S.A. 71, 3400-3404. (Prostaglandin endoperoxides. Novel transformation of arachidonic acid in human platelets).

Hamberg,M. and Samuelsson,B. (1974b). Biochem.Biophys.Res. Comm. 61, 942-949. (Prostaglandin endoperoxides VII. Novel transformations of arachidonic acid in guinea pig lungs).

Hamberg,M., Svensson,J. and Samuelsson,B. (1975). Proc. Natl.Acad.Sci.U.S.A. 72, 2994-2998. (Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides).

Hamerman,D., Stephens,M. and Barland,P. (1961). In: Inflammation and Diseases of Connective Tissue. Eds. Mills,L.C. and Moyer,J.H. p.158-168. Saunders, Philadelphia. (Comparative histology and metabolism of synovial tissue in normal and arthritic joints).

Hansen,H.S. (1972). Prostaglandins 8, 95-105. (Inhibition by indomethacin and aspirin of 15-hydroxyprostaglandin dehydrogenase in vitro).

Hart,F.D. (1976). Brit.Med.J. 1, 763-765. (History of the treatment of rheumatoid arthritis).

Huskisson,E.C., Woolf,D.L., Balme,H.W., Scott,J. and Franklyn,S. (1976). Brit.Med.J. 1, 1048-1049. (Four new anti-inflammatory drugs: responses and variations).

Hawkins,D., Pinckard,R.N., Crawford,I.P. and Farr,R.S. (1969). J.Clin.Invest. 48, 536-542. (Structural changes in human serum albumin induced by ingestion of acetylsalicylic acid).

Hench,P.S., Kendall,E.C., Slocumb,C.H. and Polley,H.F. (1949). Ann.rheum.Dis. 8, 97-104. (Effect of hormone of adrenal cortex - 17-hydroxy-11-dehydrocorticosterone; compound E - and of pituitary adrenocorticotrophic hormone on rheumatoid arthritis; preliminary report).

Hensby,C.N. (1974a). Biochim.Biophys.Acta. 348, 145-154. (Reduction of prostaglandin E_2 to prostaglandin $F_{2\alpha}$ by an enzyme in sheep blood).

Hensby,C.N. (1974b). Prostaglandins 8, 369-377. (The enzymic conversion of prostaglandin D_2 to prostaglandin $F_{2\alpha}$).

Higgs,G.A., McCall,E. and Youlten,L.J.F. (1975). Brit.J. Pharmac. 53, 539-546. (A chemotactic role for prostaglandins released from polymorphonuclear leucocytes during phagocytosis).

Higgs,G.A., Vane,J.R., Hart,F.D. and Wojtulewski,J.A. (1974). In: Prostaglandin Synthetase Inhibitors. Eds. Robinson,H.J. and Vane,J.R. p.165. Raven Press, New York. (Effects of anti-inflammatory drugs on prostaglandins in rheumatoid arthritis).

Higgs,G.A. and Youlten,L.J.F. (1972). Brit.J.Pharmac. 44, 330P. (Prostaglandin production by rabbit polymorphonuclear leucocytes in vitro).

Hillier, K. and Dilley, S.R. (1974). Prostaglandins 5, 137-155. (Separation and radioimmunoassay of F prostaglandins using silica gel microcolumns).

Hinman, J.W. (1972). Ann.Rev.Biochem. 41, 161-178. (Prostaglandins).

Hong, S.L. and Levine, L. (1976). Proc.Natl.Acad.Sci.U.S.A. 73, 1730-1734. (Inhibition of arachidonic acid release from cells as the biochemical action of anti-inflammatory corticosteroids).

Horton, E.W. (1963). Nature 200, 892-893. (Action of prostaglandin E_1 on tissues which respond to bradykinin).

Hucker, H.B., Zacchei, A.G., Cox, S.V., Brodie, D.A. and Cantwell, N.H.R. (1966). J.Pharmac.Exp.Ther. 153, 237-249. (Studies on the absorption, distribution and excretion of indomethacin in various species).

Humphrey, J.M. and Jacques, R. (1954). J.Physiol.(Lond.) 124, 305-310. (The histamine and serotonin content of the platelets and polymorphonuclear leucocytes of various species).

Jafari, E., Saleem, A., Shaikh, B.S. and Demers, L.M. (1976). Prostaglandins 12, 829-835. (Effect of aspirin on prostaglandin synthesis by human platelets).

Janszen, F.H.A. and Nugteren, D.H. (1971). Histochemie 27, 159-164. (Histochemical localisation of prostaglandin synthetase).

Jones, R.L. (1974). Biochem.J. 139, 389-391. (Inhibitors of rabbit plasma prostaglandin A isomerase).

Jonsson, C-E. (1971). Scand.J.Plast.Reconstr.Surg. 5, 1-5. (Smooth muscle stimulating lipids in peripheral lymph after experimental burn injury).

Jouvenaz, G.H., Nugteren, D.H., Beerthuis, R.K., vanDorp, D.A. (1970). Biochim.Biophys.Acta. 202, 231-234. (A sensitive method for the determination of prostaglandins by gas chromatography with electron capture detection).

Kaley, G. and Weiner, R. (1971). Ann.N.Y.Acad.Sci. 180, 338-350. (Prostaglandin E - a potential mediator of the inflammatory response).

Kalinder, M. and Austin, K.F. (1974). Biochem.Pharmac. 23, 763-771. (Cyclic nucleotides and modulation of effector systems in inflammation).

Kantrowitz, F., Robinson, D.R., McGuire, M.B. and Levine, L. (1975). Nature 258, 737-739. (Corticosteroids inhibit prostaglandin production by rheumatoid synovia).

Kaplan, A.P., Kay, A.B. and Austen, K.F. (1972). J.Exp.Med. 135, 81-97. (A prealbumin activator of prekallikrein. III. Appearance of chemotactic activity for human neutrophils by the conversion of prekallikrein to kallikrein).

Kapp, E.M. and Coburn, A.F. (1942). J.biol.Chem. 145, 549-565. (Urinary metabolites of sodium salicylate).

Kingston, W.P. and Greaves, M.W. (1976). Prostaglandins 8, 22-31. (Factors affecting prostaglandin synthesis by rat skin microsomes).

Klein, D.C. and Raisz, L.G. (1970). Endocrinol. 86, 1436-1440. (Prostaglandins: stimulation of bone resorption in tissue culture).

Kloeze, J. (1967). In: Prostaglandins. Eds. Bergström, S. and Samuelsson, B. p.241. Almqvist and Wiksell, Stockholm. (Prostaglandins and platelet aggregation).

Kocsis, J.J., Hernandovich, J., Silver, M.J., Smith, J.B. and Ingerman, C. (1973). Prostaglandins 3, 141-144. (Duration of inhibition of platelet prostaglandin formation and aggregation by ingested aspirin or indomethacin).

Ku, E.C. and Wasvary, J.M. (1973). Fed.Proc. 32, 3302. (Inhibition of prostaglandin synthetase by Su-21524).

Kunze, H. and Vogt, W. (1971). Ann.N.Y.Acad.Sci. 180, 123-125. (Significance of phospholipase A for prostaglandin formation).

Lands, W.E.M., Lee, R. and Smith, W. (1971). Ann.N.Y.Acad.Sci. 180, 107-122. (Factors regulating the biosynthesis of various prostaglandins).

Lands, W.E.M., LeTellier, P.R., Rome, L.H. and Vanderhoek, J.Y. (1973). Adv.Biosci. 9, 15-28. (Inhibition of prostaglandin biosynthesis).

Lands, W.E.M. and Samuelsson, B. (1968). Biochim.Biophys. Acta. 164, 426-429. (Phospholipid precursors of prostaglandins).

Lee, J.B. (1973). In: The Prostaglandins. vol 1. Ed. Ramwell, P.W. Plenum Press, New York. (Renal homeostasis and the hypertensive state: a unifying hypothesis).

Lee, R.E. and Lands, W.E.M. (1972). Biochim.Biophys.Acta. 260, 203-211. (Cofactors in the biosynthesis of prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$).

Levine, L. (1973). Pharmac. Rev. 25, 293-307. (Antibodies to pharmacologically active molecules: Specificities and some applications of antiprostaglandins).

Levy, G. (1963). In: Salicylates. Eds. Dixon, A.St.J., Martin, B.K., Smith, M.J.H. and Wood, P.H.N. p.17. Churchill, London.

Levy, G. and Leonards, J.R. (1966). In: The Salicylates. Eds. Smith, M.J.H. and Smith, P.K. p.5-48. Interscience, London.

Lewis, A.J., Nelson, D.J. and Sugrue, M.F. (1974). Brit. J. Pharmac. 50, 468P. (Potentiation by PGE_1 and arachidonic acid of oedema in the rat paw induced by various phlogistic agents).

Lewis, G.P. and Piper, P.J. (1975). Nature 254, 308-311. (Inhibition of release of prostaglandins as an explanation of some of the actions of anti-inflammatory corticosteroids).

Lim, R.K.S., Guzman, F., Rodgers, D.W., Goto, K., Braun, C., Dickerson, G.D. and Engle, R.J. (1964). Arch. Int. Pharmac. Ther. 152, 25-58. (Site of action of narcotic and non-narcotic analgesics determined by blocking bradykinin-evoked visceral pain).

Maddox, I.S. (1973). Biochim. Biophys. Acta. 306, 74-81. (The role of copper in prostaglandin synthesis).

Matschinsky, F.M., Shanahan, D. and Ellerman, J. (1974). Anal. Biochem. 60, 188-200. (An improved procedure for the enzymatic analysis of prostaglandins in the nanogram range).

McDonald-Gibson, R.G., Flack, J.D. and Ramwell, P.W. (1973). Biochem. J. 132, 117-120. (Inhibition of prostaglandin biosynthesis by 7-oxa- and 5-oxa-prostaglandin analogues).

Melmon, K.L., Webster, M.E., Goldfinger, S.E. and Seegmiller, J.E. (1967). Arth. Rheum. 10, 13-20. (The presence of a kinin in niflammatory synovial effusion from arthritides of varying etiologies).

Milton, A.S. and Wendlandt, S. (1971). J. Physiol. 218, 325-336. (Effects on body temperature of prostaglandins of the A, E and F series on injection into the third ventricle of unanaesthetised cats and rabbits).

Misra, D.P., Staddon, G., Powell, N., Misra, J. and Crook, D. (1974). Clin. Chim. Acta. 56, 83-89. (Lecithin-cholesterol acyltransferase activity in diabetes mellitus and the effect of insulin on these cases).

Miyamoto,T., Yamamoto,S. and Hayaishi,O. (1974). Proc.Natl.Acad.Sci.U.S.A. 71, 3645-3649. (Prostaglandin synthetase system - resolution into oxygenase and isomerase components).

Moncada,S., Ferreira,S.H. and Vane,J.R. (1973). Nature 246, 217-219. (Prostaglandins, aspirin-like drugs and the oedema of inflammation).

Moncada,S., Gryglewski,R., Bunting,S. and Vane,J.R. (1976). Nature 263, 663-665. (An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation).

Morozowich,W. (1974). J.Pharm.Sci. 63, 800-802. (Separation of PGA_2 and PGB_2 by ion-exchange liquid chromatography).

Multicentre Trial Group. (1973). Lancet i, 275-280. (Controlled trial of D(-) penicillamine in severe rheumatoid arthritis).

Needleman,P., Moncada,S., Bunting,S., Vane,J.R., Hamberg,M. and Samuelsson,B. (1976). Nature 261, 558-560. (Identification of an enzyme in platelet microsomes which generates thromboxane A_2 from prostaglandin endoperoxides).

Newkirk,J.D. and Waite,M. (1971). Biochim.Biophys.Acta. 225, 224-233. (Identification of phospholipase A_1 in plasma membranes of rat liver).

Niedermeier,W. (1965). Ann.rheum.Dis. 24, 544-548. (Concentration and chemical state of copper in synovial fluid and blood serum of patients with rheumatoid arthritis).

Nijkamp,F.P., Flower,R.J., Moncada,S. and Vane,J.R. (1976). Nature 263, 479-482. (Partial purification of rabbit aorta contracting substance-releasing factor and inhibition of its activity by anti-inflammatory steroids).

Nimni,M.E. and Bavelta,L.A. (1965). Science 150, 905-906. (Collagen defect induced by penicillamine).

Norcross,B.M. (1965). J.New Drugs 5, 259-260. (Abstr.). (Clinical effects of indomethacin in rheumatic diseases).

Nugteren,D.H., Beerthuis,R.K. and vanDorp,D.A. (1966). Rec.Trav.Chim. 85, 405-419. (The enzymic conversion of all-cis 8,11,14-eicosatrienoic acid into prostaglandin E_1).

Øie,S. and Frislid,K. (1971). Pharm.Acta.Helvetia 46, 632-636. (A fluorimetric method for direct determination of total salicylate in plasma).

Pace-Asciak, C. (1975). J.biol.Chem. 250, 2789-2794. (Prostaglandin 9-hydroxydehydrogenase activity in adult rat kidney - identification, assay, pathway and some enzyme properties).

Pace-Asciak, C. (1976). Experientia 32, 291-292. (A new prostaglandin metabolite of arachidonic acid. Formation of 6-keto-$PGF_{1\alpha}$ by the rat stomach).

Pace-Asciak, C. and Wolfe, L.S. (1970). Biochim.Biophys. Acta. 218, 539-542. (Biosynthesis of prostaglandins E_2 and $F_{2\alpha}$ from tritium-labelled arachidonic acid by rat stomach homogenates).

Panganamala, R.V., Sharma, H.M., Sprecher, H., Geer, J.C. and Cornwell, D.G. (1974). Prostaglandins 8, 3-11. (A suggested role for hydrogen peroxide in the biosynthesis of prostaglandins).

Paz, R.A. and Spector, W.G. (1962). J.Pathol.Bacteriol. 84, 83-103. (The mononuclear cell response to injury).

Phillips, H.J. (1973). In: Dye Exclusion Tests for Cell Viability. Academic Press, New York. p.406.

Pinckard, R.N., Hawkins, D. and Farr, R.S. (1968). Nature 219, 68-69. (In vitro acetylation of plasma proteins, enzymes and DNA by aspirin).

Piper, P.J. and Vane, J.R. (1969). In: Prostaglandins, Peptides and Amines. Eds. Mantegazza, P. and Horton E.W. p.15-19. Academic Press, New York. (The release of prostaglandins during anaphylaxis in guinea pig isolated lungs).

Piper, P.J. and Vane, J.R. (1971). Ann.N.Y.Acad.Sci. 180, 363-385. (the release of prostaglandins from lung and other tissues).

Pong, S.S. and Levine, L. (1976). J.Pharmac.Exp.Ther. 196, 226-230. (Prostaglandin synthetase systems of rabbit tissues and their inhibition by non-steroidal anti-inflammatory drugs).

Pugsley, D.J. and Beilin, L.J. (1974). J.Physiol. 241, 78-81P. (Analysis of prostaglandin E in renal medullary slices by gas liquid chromatography).

Rainsford, K.D. and Whitehouse, M.W. (1976). J.Pharm. Pharmac. 28, 83-86. (Concerning the merits of copper aspirin as a potential anti-inflammatory drug).

Rajan, K.T., Hill, A.G.S., Bark, A. and Whitwell, E. (1967). Ann.rheum.Dis. 26, 43-46. (Flufenamic acid in rheumatoid arthritis).

Raz, A., Stern, H. and Kenig-Wakshal, R. (1973). Prostaglandins 3, 337-352. (Indomethacin and aspirin inhibition of prostaglandin E_2 synthesis by sheep seminal vesicles microsome powder and seminal vesicle slices).

Richardson, T. (1976). J.Pharm.Pharmac. 28, 666. (Salicylates, copper complexes, free radicals and arthritis).

Riddle, J.M., Bluhm, G.B. and Barnhart, M.I. (1965). J.Reticul.Soc. 2, 420-436. (Interrelationships between fibrin, neutrophils and rheumatoid synovitis).

Robak, J., Dembinska-Kiec, A. and Gryglewski, R. (1975). Biochem.Pharmac. 24, 2057-2060. (The influence of saturated fatty acids on prostaglandin synthetase activity).

Robinson, D.R., Tashjian, A.H. and Levine, L. (1975). J.Clin.Invest. 56, 1181-1188. (Prostaglandin-stimulated bone resorption by rheumatoid synovia. A possible mechanism for bone destruction in rheumatoid arthritis).

Robinson, D.R. and McGuire, M.B. (1975). Ann.N.Y.Acad.Sci. 256, 318-329. (Prostaglandins in the rheumatic diseases).

Rose, A.J. (1975). Ph.D. Thesis, University of Bath. (Subcellular actions of non-steroidal anti-inflammatory drugs).

Rose, A.J. and Collins, A.J. (1974). Prostaglandins 8, 271-283. (The effect of pH on the production of prostaglandins E_2 and $F_{2\alpha}$ and a possible pH - dependent inhibitor).

Rosenberg, F.J., Gimber-Phillips, P.E., Groblewski, G.E., Davison, C., Phillips, D.K., Goralnick, S.J. and Cahill, E.D. (1971). J.Pharmac.Exp.Ther. 179, 410-418. (Acetylsalicylic acid: inhibition of platelet aggregation in the rabbit).

Roth, G.J. and Majerus, P.W. (1975). J.Clin.Invest. 56, 624-632. (The mechanism of the effect of aspirin on human platelets. 1. Acetylation of a particulate fraction protein).

Roth, G.J., Stanford, N. and Majerus, P.W. (1975). Proc.Natl. Acad.Sci.U.S.A. 72, 3073-3076. (Acetylation of prostaglandin synthetase by aspirin).

Rothermich, N.O. (1966). J.Amer.Med.Ass. 195, 123-128. (An extended study of indomethacin. 1. Clinical pharmacology).

Ryhage, R. and Samuelsson, B. (1965). Biochem. Biophys. Res. Comm. 19, 279-282. (The origin of oxygen incorporated during the biosynthesis of prostaglandin E_1).

Samuelsson, B. (1969). Progr. Biochem. Pharmac. 5, 109-128. (Biosynthesis of prostaglandins).

Samuelsson, B. (1970). In: Lipid Metabolism. Ed. Wakil, S.J. p.140. Academic Press, New York and London. (Structures, biosynthesis and metabolism of prostaglandins).

Samuelsson, B. (1972). Fed. Proc. 31, 1442-1450. (Biosynthesis of prostaglandins).

Schwartzman, M., Gafni, Y. and Raz, A. (1976). Eur. J. Biochem. 64, 527-534. (Properties of prostaglandin synthetase of rabbit kidney medulla).

Shaw, J.E. and Ramwell, P.W. (1968). In: Prostaglandin Symposium of the Worcester Foundation. Eds. Ramwell, P.W. and Shaw, J.E. p.55-56. Wiley, New York and London. (Inhibition of gastric secretion in rats by prostaglandin E_1).

Shen, T.Y., Windholz, T.B., Rosegay, A., Witzel, B.E., Wilson, A.N., Willet, J.D., Holtz, W.J., Ellis, R.L., Matzuk, A.R., Lucas, S.L., Stammer, C.H., Holly, F.W., Sarett, L.H., Risley, E.A., Nuss, G.W. and Winter, C.A. (1963). J. Amer. Chem. Soc. 85, 488-489. (Non-steroid anti-inflammatory agents).

Sih, C.J. and Takeguchi, C.A. (1973). In: The Prostaglandins. Ed. Ramwell, P.W. Plenum Press, New York and London. p.83-100.

Smith, A.P. (1972). In: The Prostaglandins: Progress in Research. Ed. Karim, S.S.M. p.223. MTP, Oxford.

Smith, A.P. and Cuthbert, M.F. (1973). Adv. Biosci. 9, 213-218. (The effects of inhaled prostaglandins on bronchial tone in man).

Smith, J.B. and Willis, A.L. (1971). Nature (New Biol.) 231, 235-237. (Aspirin selectively inhibits prostaglandin production in human platelets).

Smith, M.J.H. (1975). Agents and Actions 5, 315-317. (Prostaglandins and aspirin: an alternative view).

Smith, M.J.H. and Dawkins, P.D. (1971). J. Pharm. Pharmac. 23, 729-744. (Review: Salicylates and enzymes).

Smith, M.J.H., Dawkins, P.D. and McArthur, J.N. (1971). J. Pharm. Pharmac. 23, 451. (The relation between clinical anti-inflammatory activity and the displacement of L-tryptophan and a dipeptide from human serum *in vitro*).

Smith,M.J.H., Ford-Hutchinson,A.W. and Elliot,P.N.C. (1975). J.Pharm.Pharmac. 27, 473-478. (Prostaglandins and the anti-inflammatory activities of aspirin and sodium salicylate).

Smith,W.L. (1975). Prostaglandins 10, 983-990. (Flurbiprofen-sepharose chromatography of the prostaglandin synthetase from bovine seminal vesicles).

Smith,W.L. and Lands,W.E.M. (1971). J.biol.Chem. 21, 6700-6704. (Stimulation and blockade of prostaglandin biosynthesis).

Sorenson,J.R.J. (1976). J.Med.Chem. 19, 135-148. (Copper chelates as possible active forms of the antiarthritic agents).

Steiner,M. (1970). Experientia 26, 786-789. (Platelet protein synthesis studied in a cell-free system).

Stone,K.J., Mather,S.J. and Gibson,P.P. (1975). Prostaglandins 10, 241-251. (Selective inhibition of prostaglandin biosynthesis by gold salts and phenylbutazone).

Struijk,C.B., Beerthuis,R.K., Pabon,H.J.J. and vanDorp,D.A. (1966). Rec.Trav.Chim. 85, 1233-1250. (Specificity in the enzymic conversion of polyunsaturated fatty acids into prostaglandins).

Swinson,D.R., Bennett,A. and Hamilton,E.B.D. (1976). In: The Role of Prostaglandins in Inflammation. Ed. Lewis,G.P. p.41-45. Hans Huber, Bern, Stuttgart and Vienna. (Synovial prostaglandins in joint disease).

Tai,H-H., Tai,C.L. and Hollander,C.S. (1976). Biochem.J. 154, 257-264. (Biosynthesis of prostaglandins in rabbit kidney medulla. Properties of prostaglandin synthetase).

Takeguchi,C. and Sih,C.J. (1972). Prostaglandins 2, 169-184. (A rapid spectrophotometric assay for prostaglandin synthetase: application to the study of non-steroidal anti-inflammatory agents).

Tashjian,A.H., Voelkel,E.F., Levine,L. and Goldhaber,P. (1972). J.Exp.Med. 136, 1329-1343. (Evidence that the bone resorption-stimulating factor produced by mouse fibrosarcoma cells is prostaglandin E_2. A new model for the hypercalcaemia of cancer).

Tashjian,A.H., Voelkel,E.F., McDonough,J. and Levine,L. (1975). Nature 258, 739-741. (Hydrocortisone inhibits prostaglandin production by mouse fibrosarcoma cells).

Tauber, A.I., Kalinder, M., Stechshulte, D.J. and Austen, K.F. (1973). J.Immunol. 111, 27-32. (Immunologic release of histamine and slow reacting substance of anaphylaxis from human lung. V. Effects of prostaglandins on release of histamine).

Thomas, G. and West, G.B. (1973). J.Pharm.Pharmac. 25, 747-748. (Prostaglandins as regulators of bradykinin responses).

Turner, S.R., Campbell, J.A. and Lynn, W.S. (1975). J.Exp.Med. 141, 1437-1441. (Polymorphonuclear leukocyte chemotaxis toward oxidised lipid components of cell membranes).

van Dorp, D.A. (1967). Progr.Biochem.Pharmac. 3, 71-79. (Aspects of the biosynthesis of prostaglandins).

van Dorp, D.A., Beerthuis, R.K., Nugteren, D.H. and Vonkeman, H. (1964a). Biochim.Biophys.Acta. 90, 204-207. (The biosynthesis of prostaglandins).

van Dorp, D.A., Beerthuis, R.K., Nugteren, D.H. and Vonkeman, H. (1964b). Nature(Lond.) 203, 839-841. (Enzymatic conversion of all-cis-polyunsaturated fatty acids into prostaglandins).

Vane, J.R. (1971). Nature(New Biol.) 231, 232-235. (Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs).

Vane, J.R. (1972). Hosp.Pract. 7, 61-71. (Prostaglandins and the aspirin-like drugs).

Vonkeman, H. and van Dorp, D.A. (1968). Biochim.Biophys. Acta. 164, 430-432. (The action of prostaglandin synthetase on 2-arachidonyl-lecithin).

Walker, J.R., Smith, M.J.H. and Ford-Hutchinson, A.W. (1976). J.Pharm.Pharmac. 28, 745-747. (Prostaglandins and leucotaxis).

Wallach, D.P. and Daniels, E.G. (1971). Biochim.Biophys. Acta. 231, 445-457. (Properties of a novel preparation of prostaglandin synthetase from sheep seminal vesicles).

Weber, K. and Osborn, M. (1969). J.biol.Chem. 244, 4406-4412. (The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis).

Wegelius, O., Laine, V., Lindström, B. and Klockars, M. (1970). Acta.Med.Scand. 187, 539-544. (Fistula of the thoracic duct as immunosuppressive treatment in rheumatoid arthritis).

Weissmann, G. (1964). Lancet ii, 1373-1375. (Lysosomes, autoimmune phenomena and diseases of connective tissues).

Weissman, G. (1972). N.Engl.J.Med. 286, 141-147. (Lysosomal mechanisms of tissue injury in arthritis).

Whitehouse, M.W. (1965). Prog.Drug.Res. 8, 321-429. (Some biochemical and pharmacological properties of anti-inflammatory drugs).

Whitehouse, M.W. (1968). In: Chemical Biology of Inflammation. Eds. Houck, J.C. and Forscher, B.K. Pergammon Press, London. (The molecular pharmacology of anti-inflammatory drugs: some possible mechanisms of action at the biochemical level).

Whitehouse, M.W. (1974). In: Anti-inflammatory Agents. Vol.2. Eds. Scherrer, R.A. and Whitehouse, M.W. p.1-31. Academic Press, New York.

Williams, T.J. and Morley, J. (1973) Nature, 246, 215-217. (Prostaglandins as increased potentiators of increased permeability in inflammation).

Willis, A.L. (1969). In: Prostaglandins, Peptides and Amines. Eds. Mantegazza, P. and Horton, E.W. p.31-38. Academic Press, New York. (Release of histamine, kinin and prostaglandins during carrageenin-induced inflammation in the rat).

Willis, A.L. (1973). Circulation 48, (Suppl.4) 55. (Platelet synthesis of pro-aggregating material from arachidonate and its blockade by aspirin).

Willis, A.L., Davison, P., Ramwell, P.W., Brocklehurst, W.E. and Smith, B. (1972). In: Prostaglandins in Cellular Biology. Eds. Ramwell, P.W. and Phariss, B.B. p.227-259. Plenum Press, New York. (Release and actions of prostaglandins in inflammation and fever: Inhibition by anti-inflammatory and anti-pyretic drugs).

Willoughby, D.A., Giroud, G.P., Di Rosa, M. and Velo, G.P. (1973). In: Prostaglandins and Cyclic AMP. Eds. Kahn, R.H. and Lands, W.E.M. p.187-206. Academic Press, New York. (The control of the inflammatory response with special references to the prostaglandins).

Winter, A.C., Risley, A.E. and Nuss, G.W. (1963). J.Pharmac. Exp.Ther. 141, 369-376 (Anti-inflammatory and antipyretic activities of indomethacin, 1-(p-chloro-benzoyl) -5- methoxy -2-methyl-indole-3-acetic acid).

Wlodawar, P. and Samuelsson, B. (1973). J.biol.chem. 248, 5673-5678. (On the organisation and mechanism of prostaglandin synthetase).

Woodbury, D.M. (1965). In: The Pharmacological Basis of Therapeutics. 3rd Edn. Ed. Collier, H.O.J. p.329. Macmillan, London.

Yoshimoto,A., Ito,H. and Tomita,K. (1970). J.Biochem. 68, 487-499. (Cofactor requirements of the enzyme synthesising prostaglandin in bovine seminal vesicles).

Youdim,M.B.H. and Woods,H.F. (1975). Biochem.Pharmac. 24, 317-323. (The influence of tissue environment on the rates of metabolic processes and the properties of enzymes).

Yu,D.T.Y. and Peter,J.B. (1974). Sem.Arth.Rheum. 4, 25-52. (Cellular immunological aspects of rheumatoid arthritis).

Zia,P. and Horton,R. (1973). Prostaglandins 4, 543-551. (A chromatographic technique (Sephadex LH-20) for the separation of major prostaglandins).

Ziboh,V.A. (1973). J.Lipid Res. 14, 377-384. (Biosynthesis of prostaglandin E_2 in human skin: subcellular localization and inhibition by unsaturated fatty acids and anti-inflammatory drugs).

Zurier,R.B. and Quagliata,F. (1971). Nature 234, 304-305. (Effects of PGE_2 on adjuvant arthritis).

B I B L I O G R A P H Y

Prostaglandin Synthetase Inhibitors. Eds. Robinson, H.J. and Vane, J.R. Raven Press, New York. (1974).

Prostaglandins in Cellular Biology. Eds. Ramwell, P.W. and Phariss, B.B. Plenum Press, New York (1972).

Lipid Metabolism. Ed. Wakil, S.J. Academic Press, New York and London (1970).

Prostaglandins, Peptides and Amines. Eds. Mantegazza, P. and Horton, E.W. Academic Press, New York (1969).

Prostaglandin Symposium of the Worcester Foundation. Eds. Ramwell, P.W. and Shaw, J.E. Wiley, New York (1968).

Future Trends in Inflammation. Eds. Velo, G.P., Willoughby, D.A. and Giroud, J.P. Piccin Medical Books, Padua. (1974).

The Prostaglandins: Progress in Research. Ed. Karim, S.S.M. MTP, Oxford (1972).

The Prostaglandins, Volume 1. Ed. Ramwell, P.W. Plenum Press, New York and London (1973).

Prostaglandins and Cyclic AMP. Eds. Kahn, R.H. and Lands, W.E.M. Academic Press, New York (1973).

Prostaglandins. von Euler, U.S. and Eliasson, R. Academic Press, New York and London (1967).

The Chemical Pathology of Rheumatoid Arthritis. West, H.F. Charles C. Thomas, Springfield, Illinois, U.S.A. (1970).

Anti-inflammatory Agents. Vols. 1 and 2. Eds. Scherrer, R.A. and Whitehouse, M.W. Academic Press, New York. (1974).

The Inflammatory Process. Second Edition. Eds. Zweifach, B.W., Grant, L. and McCluskey, R.T. Academic Press, New York, San Francisco and London. (1974).

Inflammation and Diseases of Connective Tissue. Eds. Mills, L.C. and Moyer, J.H. Saunders, Philadelphia (1961).

The Role of Prostaglandins in Inflammation. Proceedings of a Workshop held during the VIIIth European Rheumatology Congress, Helsinki, 1975. Ed. Lewis, G.P. Hans Huber, Bern, Stuttgart and Vienna (1976).

Salicylates. Eds. Dixon, A. St. J., Martin, B.K., Smith, M.J.H. and Wood, P.H.N. Churchill, London (1963).

The Salicylates. Eds. Smith, M.J.H. and Smith, P.K. Interscience Publishers, New York, (1966).